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ON ANTIDIABETIC AGENTS AND THE VASCULATURE

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TILL MIN FAMILJ

'The right dose differentiates a poison from a useful medicine'
- Paracelsus (1493–1541)

ABSTRACT

Diabetes is rapidly increasing worldwide, and the number of patients suffering from diabetes is projected to rise with 50 % over the next 25 years, then affecting almost 600 million adults. Type 2 diabetes comprises 90-95 % of all people with diabetes, and they constitute a patient group that carries a high burden of cardiovascular disease. Patients with type 2 diabetes suffer a 2-4 times higher risk for myocardial infarction and stroke than healthy persons. In addition to this, diabetic patients have an increased atherosclerotic burden. Endothelial dysfunction is thought to be an early and important predictor of atherosclerosis and cardiovascular disease, and in people with type 2 diabetes endothelial dysfunction is a common finding. Also, diabetic patients have an increased risk of restenosis and late stent thrombosis upon surgical intervention to treat atherosclerosis, complications that are related to a defective re-growth of the endothelial cells. The relationship between hyperglycemia and macrovascular complications is still uncertain, at least in terms of the possibility of reducing cardiovascular events solely by improving glycemic control. The importance of finding other strategies to improve cardiovascular outcome in type 2 diabetes patients has consequently emerged. My aim with this thesis has therefore been to investigate how agents used for type 2 diabetes management directly affect the vasculature under normal and simulated diabetic conditions.

We studied the effect of antidiabetic agents on endothelial cell viability, regeneration and apoptosis in three in vitro studies. We found that a number of agents could induce proliferation under normal and hyperglycemic conditions, and protect endothelial cells from free fatty acid-induced apoptosis. We studied two antihyperglycemic agents', metformin and exendin-4, lipoprotective effects in more detail. We found that exendin-4 and metformin protected endothelial cells against lipoapoptosis by modulating pro-apoptotic kinases. Exendin-4 and metformin were also able to activate intracellular survival pathways and to improve endothelial nitric oxide synthase dysfunction. In our last study, we investigated the effect of exendin-4 on endothelial cells and smooth muscle cells in vivo by using a rat model of vascular injury, where the endothelium is denuded in the carotid artery; we found that exendin-4 selectively targeted the smooth muscle cells and decreased the injury-induced intimal hyperplasia. Treatment with exendin-4 did not affect the re-growth of the endothelial cells, but on the other hand improved arterial wall elasticity, suggesting that the re-grown endothelial cells were better functioning in exendin-4-treated animals.

In conclusion, we show that drugs used in the management of type 2 diabetes exert direct positive effects on the vasculature. This might be of clinical benefit for patients suffering from diabetes by limiting the adverse consequences of the macrovascular complications of type 2 diabetes, as dysfunction of endothelial cells and smooth muscle cells is believed to contribute to premature development of atherosclerosis. Our findings might also be of therapeutic benefit for diabetic patients undergoing revascularization to treat atherosclerosis, since restenosis and late stent thrombosis are overrepresented and serious complications among these patients.

SVENSK SAMMANFATTNING

Antalet personer som lider av diabetes ökar snabbt världen över och prevalensen av diabetes är beräknad att öka med 50 % under de nästkommande 25 åren och kommer då att drabba ungefär 600 miljoner vuxna. Personer med typ 2 diabetes utgör 90-95 % av det totala antalet diabetiker i världen och de är en patientgrupp med en förhöjd risk för att utveckla hjärt- och kärlsjukdomar. Jämfört med en frisk person så har en typ 2 diabetiker en 2-4 gånger högre risk att insjukna i hjärtinfarkt eller stroke, dessutom har diabetespatienter även en ökad börda av ateroskleros. Endoteldysfunktion tros vara en tidig indikator för utvecklandet av ateroskleros samt kardiovaskulär sjukdom och endoteldysfunktion ses ofta hos typ 2 diabetes patienter. Därutöver så är det vanligare att diabetiker dabbas av komplikationer så som återförträngning av kärlet och trombos efter kirurgiska interventioner riktade mot att behandla åderförkalkning. Dessa komplikationer är associerade till en defekt återväxt av endotelcellerna. Om ett förhållande finns mellan hyperglykemi och makrovaskulära komplikationer är inte helt klarlagt, åtminstone när det gäller möjligheten att minska risken för kardiovaskulära händelser enbart genom att förbättra den glykemiska kontrollen. Därför är det viktigt att hitta andra strategier för att minska uppkomsten av kardiovaskulära komplikationer hos typ 2-diabetespatienter. Syftet med denna avhandling har därför varit att undersöka hur läkemedel som används inom behandlingsramen för typ 2-diabetes direkt påverkar cellerna i våra kärl under normala och simulerade diabetiska förhållanden.

Vi studerade effekten av antidiabetiska läkemedel på endotelcells viabilitet, regeneration och celldöd *in vitro* i tre delstudier. Vi fann att ett antal läkemedel positivt påverkade celldelningen av endotelceller vilka var exponerade för både normal och hög sockernivå, samt skyddade endotelceller från celldöd inducerad av en mättad fettsyra. Vi undersökte även de två antidiabetiska substanserna exendin-4 och metformin lite närmare och från dessa studier så drog vi slutsatsen att de båda skyddade endotelceller från fettsyreinducerad celldöd genom att minska aktiviteten av proapoptotiska proteinkinaser. Exendin-4 och metformin aktiverade även skyddande cellsignaleringsvägar och förbättrade funktionen av endotelcellernas kväveoxidsyntas. I vår fjärde delstudie utredde vi effekten av exendin-4 på endotel- och glattmuskelceller *in vivo* vid kärlskada med hjälp av en råttmodell där endotelet skrapas bort i karotisartären. Våra fynd visade att exendin-4 selektivt påverkar glattmuskelceller samt minskade tillväxten av intiman. Behandling med exendin-4 påverkade inte återväxten av endotelcellerna, men däremot förbättrades elasticiteten i kärlväggen, vilket kan tyda på att exenidin-4 behandling genererar mer välfungerande endotelceller.

För att summera, vi visar i denna avhandling att läkemedel som används i behandlingen av typ 2-diabetes utövar positiva effekter på kärlsystemet. Detta kan vara till klinisk nytta för patienter som lider av diabetes då det kan bidra till att begränsa konsekvenserna av de kardiovaskulära komplikationerna vid typ 2-diabetes, eftersom dysfunktion av endotelceller tros bidra till en prematur utveckling av ateroskleros. Våra resultat kan också vara av terapeutisk nytta för diabetespatienter som genomgår revaskularisering i syfte att behandla åderförkalkning, eftersom potentiellt dödliga komplikationer såsom förträngning och trombos är överrepresenterade inom denna patientgrupp.

LIST OF PUBLICATIONS

I. **Linnéa Eriksson**, Özlem Erdogdu, Thomas Nyström, Qimin Zhang and Åke Sjöholm.
Effects of some anti-diabetic and cardioprotective agents on proliferation and apoptosis of human coronary artery endothelial cells
Cardiovasc Diabetol. 2012 Mar 21;11:27

II. Özlem Erdogdu*, **Linnéa Eriksson***, Hua Xu, Åke Sjöholm, Qimin Zhang and Thomas Nyström (*Authors contributed equally to this work)
Exendin-4 protects endothelial cells from lipoapoptosis by PKA, PI3K, eNOS, p38 MAPK, and JNK pathways
J Mol Endocrinol. 2013 Mar 18;50(2):229-41

III. **Linnéa Eriksson**, Åke Sjöholm, and Thomas Nyström
Activation of AMP-activated protein kinase protects human coronary artery endothelial cells against diabetic lipoapoptosis
(Manuscript)

IV. **Linnéa Eriksson**, Robert Saxelin, Samuel Röhl, Joy Roy, Kenneth Caidahl, Thomas Nyström, Ulf Hedin, Åke Sjöholm, and Anton Razuvaev
GLP-1 receptor agonist exendin-4 reduces intimal hyperplasia via direct effects on smooth muscle cells in a non-diabetic rat model of arterial injury
(Submitted)

Other publications not included in this thesis:

Özlem Erdogdu, **Linnéa Eriksson**, Thomas Nyström, Åke Sjöholm, and Qimin Zhang
Exendin-4 restores glucolipotoxicity-induced gene expression in human coronary artery endothelial cells.
Biochem Biophys Res Commun. 2012 Mar 23;419(4):790-5.

CONTENTS

LIST OF ABBREVIATIONS

ACCORD	Action to Control Cardiovascular Risk in Diabetes
ADA	American Diabetes Association
ADMA	Asymmetric dimethyl arginine
ADVANCE	Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation
AGE	Advanced glycosylation end product
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AMPK	AMP-activated protein kinase
ARB	Angiotensin II receptor blocker
AT_1	Angiotensin II receptor type 1
BH_4	Tetrahydrobiopterin
BMI	Body mass index
BSA	Bovine serum albumin
CABG	Coronary artery bypass grafting
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
DAG	Diacylglycerol
DCCT	Diabetes Control and Complications Trial
DES	Drug eluting stent
DPP-4	Dipeptidyl peptidase-4
EASD	European Association for the Study of Diabetes
ECL	Enhanced chemiluminescence
EDHF	Endothelial derived hyperpolarizing factor
EDIC	Epidemiology of Diabetes Interventions and Complications
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
EXAMINE	Examination of Cardiovascular Outcomes with AlogliptIn Versus Standard of Care in Patients with Type 2 Diabetes Mellitus and Acute Coronary Syndrome
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFA	Free fatty acid
FMD	Flow mediated dilation
FMN	Flavin mononucleotide
FREEDOM	Future Revascularization Evaluation in patients with Diabetes mellitus: Optimal management of Multivessel disease
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide-1
GLP-1R	Glucagon-like peptide 1 receptor
GLUT	Glucose transporter
HCAEC	Human coronary artery endothelial cell

HDL	High-density lipoprotein
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular Adhesion Molecule 1
IKK-β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IRS-1	Insulin receptor substrate-1
JNK	c-Jun *N*-terminal kinase
K_{ATP}	ATP-dependent potassium channel
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
oxLDL	Oxidized low-density lipoprotein
PAD	Peripheral artery disease
PAI-1	Plasminogen activator inhibitor -1
PCI	Percutaneous coronary intervention
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PGI_2	Prostacyclin
PI3K	Phosphatidylinositol-3-OH kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PPAR	Peroxisome proliferator activated receptor
PTCA	Percutaneous transluminal coronary angioplasty
RAGE	Advanced glycosylation end product receptor
RECORD	Rosiglitazone Evaluated for Cardiovascular Outcomes and Regulation of Glycemia in Diabetes
ROS	Reactive oxygen species
SAVOR-TIMI	The Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus - Thrombolysis in Myocardial Infarction
SDIS	The Stockholm Diabetes Intervention Study
SMC	Smooth muscle cell
SUR	Sulfonylurea receptor
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TNF-α	Tumor necrosis factor-α
TZD	Thiazolidinedione
UKPDS	The UK Prospective Diabetes Study
VADT	Veterans Affairs Diabetes Trial
VCAM-1	Vascular cell adhesion molecule 1
WHO	World Health Organization

1 INTRODUCTION

1.1 DIABETES

Diabetes mellitus is a heterogeneous and complex group of disorders, characterized by hyperglycemia [1]. According to the World Health Organization (WHO) is diabetes diagnosed if the fasting plasma glucose is > 7 mmol/l on two different occasions or if the plasma glucose concentration is > 11.1 mmol/l two hours following an oral glucose tolerance test [2]. The American Diabetes Association (ADA) divides diabetes into four different categories based on etiology and clinical features: 1) Type 1 diabetes mellitus (T1DM), which manifests with insulin deficiency due to β-cell destruction. 2) Type 2 diabetes mellitus (T2DM), characterized by a progressive insulin secretory defect and insulin resistance. 3) Gestational diabetes, diagnosed during pregnancy, and 4) other specific types of diabetes, which might be due to genetic defects in β-cell function or insulin action, disease in the exocrine pancreas, or induced by drugs- or chemicals [3].

Diabetes is rapidly increasing worldwide, and it is estimated that 382 million adults suffer from the disease today, a number that is projected to rise with more than 50 % in less than 25 years, affecting then almost 600 million adults [4]. The global health expenditure in order to treat diabetes and its complications was estimated to 548 billion US dollars during 2013. However, there are substantial differences between countries and regions on the health spending for treating diabetes. This is clearly emphasized by the fact that 80 % of all people with diabetes lives in low- and middle income countries, but only 20 % of the global health expenditure for treatment of diabetes was spent here last year [4].

1.1.1 Type 2 diabetes

T2DM comprises 90- 95 % of all people with diabetes. It is a heterogeneous disorder but characteristic features are peripheral insulin resistance and a relative insulin deficiency due to impaired β-cell function, which eventually leads to fasting hyperglycemia, and overt T2DM [1, 3]. Although the etiology of the disease is not completely known, T2DM is associated with risk factors such as obesity, physical inactivity, family history of diabetes, advancing age, hypertension, dyslipidemia, prior gestational diabetes, and is more frequently seen in certain ethnicities [3, 4]. The disease might go unnoticed for several years since glycemia gradually increases, and it is often not diagnosed until complications due to the disease have already developed. Clinical management of T2DM consists of a combination of lifestyle interventions with changes in diet and physical activity, addition of one or more anti-diabetic agent, and if the glycemia is poorly controlled, eventually also insulin injections [3, 4]. The increase in T2DM is not only contained in the adult population; at the same time as childhood obesity is increasing, T2DM emerges as a new form of pediatric diabetes. T2DM was in the past rarely seen in young individuals (under 20 years of age), and all diabetic children were then assumed to suffer from T1DM. Today, however, studies from both China [5] and the U.S. [6] show that the prevalence of T2DM is rapidly increasing and in some ethnic groups the incidence of T2DM in young people has already surpassed that of T1DM [7, 8].

1.1.2 The metabolic syndrome

The metabolic syndrome refers to a clustering of cardiovascular risk factors, and it was first defined by Reaven in 1988 [9]. After this the WHO, the International Diabetes Federation and others have come up with their own criteria of the metabolic syndrome. There is not yet a consensus definition in place, making it hard to compare between studies and to identify individuals at risk. This has generated criticism from the European Association for the Study of Diabetes (EASD) and the ADA, which -- in a joint statement in 2005 -- suggested that the term "metabolic syndrome" should be carefully used until its status as a "syndrome" has been verified with more research [10]. However, the risk factors that are most commonly mentioned when discussing the metabolic syndrome are insulin resistance (impaired response of peripheral tissues to insulin [11]) or impaired fasting glucose, obesity (BMI or waist circumference), dyslipidemia (abnormal levels of triglycerides and/or HDL cholesterol) and hypertension. If an individual has three or more of these risk factors, he or she is considered to have the metabolic syndrome and thus an 1.5-3 fold increased risk of cardiovascular disease and five times higher risk to develop T2DM over the next five to ten years [12, 13]. As a matter of fact, insulin resistance *per se* is an independent risk factor for developing cardiovascular disease [14, 15].

1.1.3 Complications of diabetes

Diabetes is a leading cause of cardiovascular disease, blindness, kidney failure, and lower-limb amputation, in almost all high-income countries. This trend will probably be followed by low- and middle-income countries as the prevalence of diabetes increases worldwide [4]. Although microvascular complications are the most common cause of morbidity in diabetic patients, it is the macrovascular complications that account for the most common cause of mortality, where myocardial infarction (MI) and stroke account for ~ 80 % of all deaths [15]. In individuals with T2DM, the etiology for up to 75 % of the mortality is atherosclerotic cardiovascular disease [16]. In T1DM, hyperglycemia seems to be more important for the development of both micro-and macrovascular complications than in T2DM, where the picture is more complex [17].

1.1.3.1 Microvascular complications

Diabetic retinopathy is probably the most common complication of diabetes, and the strongest predictor for the disease is the duration of diabetes. It is caused by hyperglycemia in combination with hypertension and dyslipidemia. Diabetic retinopathy is the leading cause of blindness in the adult population between 20-74 years of age [1, 18].

Diabetic nephropathy is seen in 20-40 % of the diabetic patients and it is the leading cause of chronic kidney disease needing dialysis or kidney transplantation in the U.S. [18]. Like other diabetic microvascular complications, there is a strong association between glucose control and the risk of developing diabetic nephropathy. This condition is associated with changes in glomeruli and when the nephropathy is fully developed it is characterized by Kimmelstiel–Wilson nodules. These lesions are not seen in any other kidney disease, suggesting that they are specifically caused by chronic hyperglycemia seen in diabetic patients. When these changes are established, several other factors can affect the clinical outcome, especially blood pressure [19].

Hyperglycemia in combination with high blood pressure can lead to damage of the nerves (neuropathy), with the feet being most commonly affected. Neuropathies are heterogeneous and with diverse clinical manifestations. Neuropathies are common late complications of diabetes, and patients often experience pain, a tingling feeling and numbness. Numbness might complicate things further since wounds might go unnoticed, leading to foot deformations, ulcerations and infections, which in a worst case scenario lead to amputation [1, 4, 18]. The overall risk of amputation is 40 times higher in diabetics than in non-diabetic patients [1]

1.1.3.2 Macrovascular complications

Patients with T2DM suffer an increased risk for MI and stroke, a risk that is equal to patients who have already suffered a previous MI and 2-4 times higher than a person without a previous MI or T2DM [20]. In addition to this, diabetic patients have an increased atherosclerotic burden which can cause obstruction of arteries, and not only in the coronary circulation. Also the more peripheral arteries are affected, which can lead to vascular occlusion in the extremities, known as peripheral artery disease (PAD). The occlusions might cause symptoms such as pain, and cramping in the legs, due to the ischemia and lactic acid build up. PAD is a marker for systemic vascular disease and a major risk factor for lower extremity amputation [21].

In general, microvascular complications are caused by prolonged exposure to hyperglycemia, and the link between intensified glucose control and reduced complications is well supported in numerous studies for T1DM (DCCT and SDIS) and for T2DM (UKPDS, ACCORD, ADVANCE, VADT). This is in contrast to hyperglycemia and macrovascular complications, where the relationship is still uncertain, at least in terms of the possibility of reducing cardiovascular events solely by improving glycemic control [22-28]. The only randomized controlled trial that so far has been able to decrease macrovascular events and mortality within the trial period, is the STENO-2 study. Here the intervention protocol targeted all known cardiovascular risk factors, such as hyperglycemia, hypertension, dyslipidemia, overweight, physical inactivity, smoking and diet [29, 30]. On the other hand, the UKPDS (T2DM) and DCCT/EDIC (T1DM) studies did show a significant reduction of macrovascular complications in the post-trial period, even though the glycemic differences were lost [31, 32], and in speculation this was termed a “legacy effect”. However, since there is no clear cut association between hyperglycemia and macrovascular complications, it is of interest to consider whether and how drugs used against diabetes directly affect the vasculature.

1.2 THE VASCULATURE

1.2.1 Overview

Blood vessels can be divided into five different groups: **1)** the arteries, thick-walled vessels which carry the blood away from the heart. **2)** The arterioles are smaller branches of the arteries with muscular walls that deliver blood into **3)** the capillaries. The capillaries are small and their vessel walls consist of only one cell layer in order to allow for efficacious exchange of molecules between the blood and tissues and between blood and air in the lung tissue. The capillaries then unite and form **4)**

venules, that have a slightly thicker vessel wall and begin the transport of the blood back to the heart. Venules then merge and form larger vessels, *viz.* **5)** the veins, which continue the transport of blood until it is returned to the heart [33, 34].

The macrovessels (arteries, arterioles and veins) are made up of three layers (tunics), separated by elastic membranes (depicted in figure 1). The innermost tunica, the intima, is a single layer of endothelial cells lining the internal lumen of the vessel. The media, the thickest layer, which constitutes of smooth muscle cells, and the outermost membrane, the adventitia, made up of supportive connective tissue, mast cells, fibroblasts, nerve endings and microvessels (*vasa vasorum*) [33-35].

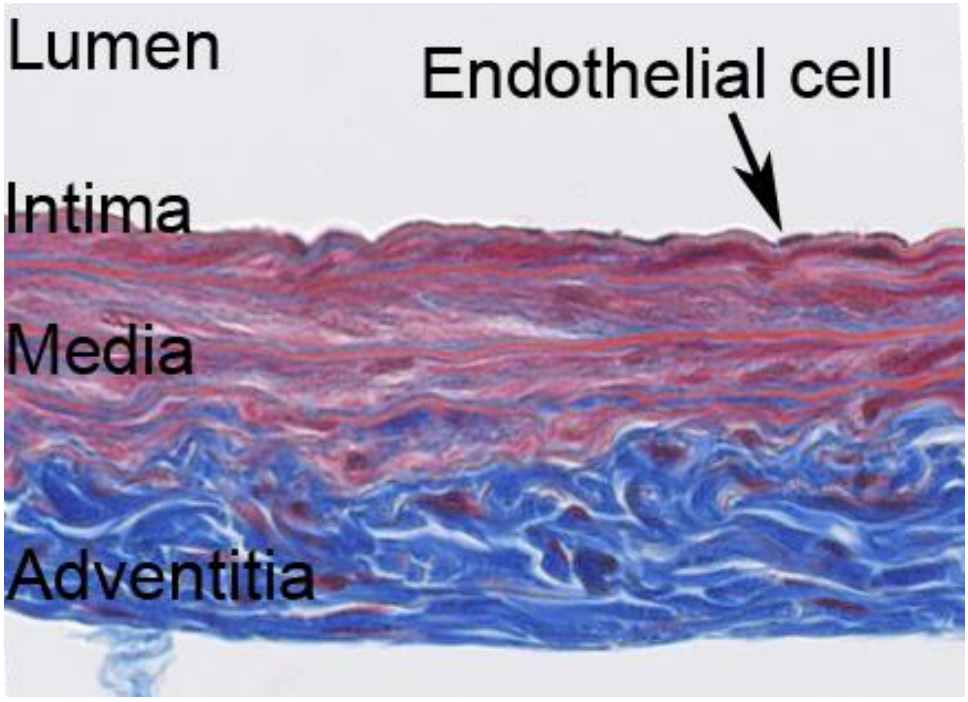

Figure 1. **Cross section of the rat common carotid artery.** The common carotid artery has been stained with Masson Trichrome, where blue staining represents collagen, red represents muscle and, hematoxylin staining identifies the nuclei.

1.2.2 Endothelial cells

The endothelial cells constitute the endothelium, a single-cell inner lining of blood vessels, which is situated as a barrier between the circulating blood and the vascular smooth muscle cells. The endothelium is the main regulator of vascular wall homeostasis by controlling the vascular permeability of constituents in plasma. It also protects the vessels from activation of clotting and proinflammatory factors by regulating platelet and leukocyte adhesion. Additionally, it participates in the regulation of vascular tone, blood flow and blood pressure by releasing vasodilating mediators such as nitric oxide (NO), endothelial derived hyperpolarizing factor (EDHF), and prostacyclin (PGI_2), and vasoconstrictors such as endothelin-1 (ET-1) and angiotensin-II [36, 37].

1.2.2.1 Endothelial nitric oxide synthase and nitric oxide production

The most potent vasodilator in the body is NO, which is synthesized when L-arginine is converted into L- citrulline by the enzyme nitric oxide synthase (NOS). There are several different isoforms of NOS, although all are homologous. The NOS family can be divided into two different categories, the constitutive and the inducible NOS, which differ in their activities and regulation. The constitutive category includes neuronal NOS (also known as NOS I) and endothelial NOS (also known as NOS III), which are abundantly expressed. These isoforms are regulated by the calcium-binding protein calmodulin (CaM), which means that they are inactive until the intracellular level of calcium rises and forms a complex with CaM. The calcium-CaM complex then binds to NOS, resulting in its activation. Inducible NOS (also known as NOS II) is CaM-independent. Its expression is dependent on inflammatory cytokines and upon activation inducible NOS produces up to a thousand times more NO than endothelial NOS (eNOS) [38, 39].

The vascular tone is controlled by eNOS producing NO in response to several stimuli, *e.g.* insulin, shear stress, bradykinin and acetylcholine [36]. eNOS activity is determined by several cofactors, such as tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), for its function [40]. Upon production diffuses NO from the endothelial cells to the smooth muscle cells (SMC), where it activates soluble guanylyl cyclase, resulting in the production of cyclic GMP (cGMP). cGMP activates a cGMP-dependent protein kinase (PKG). This leads to an increased displacement of calcium in the cytosol, which inhibitits the contractile machinery in SMCs, causing them to relax thus dilating the vessel [41]. In addition to this, NO plays an essential role in the regulation of endothelial function [42] and has been shown to be involved in endothelial cell growth, migration, apoptosis and angiogenesis [43-45]. Recently, an alternative pathway for NO formation in mammals have been described, involving the reduction of inorganic nitrate, which was long believed to merely be an undesired dietary constituent and an inert product formed upon oxidation of NO, to nitrite which can be further reduced to NO. This pathway works in parallel to the oxygen-dependent classical pathway described above, but -- more importantly -- when oxygen availability and NOS activity are reduced, *e.g.* during an ischemic event, nitrite reduction to NO becomes more pronounced. This pathway can therefore be viewed as a back‑up system to ensure that there is sufficient NO formation even when oxygen supply is limited [46].

1.2.3 Smooth muscle cells

SMCs reside underneath the endothelium, and are under normal circumstances maintained in a differentiated contractile state. Their principal function is contraction and regulation of blood vessel diameter, maintenance of blood pressure, and blood flow distribution [47]. SMCs have low turnover in their differentiated state, which is also controlled by the endothelial cells through their production of NO. NO is well-known to inhibit SMC proliferation and migration [48]. However, SMCs can dedifferentiate into a synthetic phenotype and then exhibit high rates of proliferation, migration and production of extra-cellular matrix components. The conversion of SMCs to a synthetic phenotype is an early event in many cardiovascular diseases, such as atherosclerosis, restenosis and abdominal aortic aneurysm. Intriguingly, it has been

suggested, due to the increased risk for cardiovascular complications in T2DM, that a "diabetic phenotype" of SMCs may exist, and that hyperglycemia and hyperinsulinemia might have direct effects also on the SMCs in the vessel wall [49].

1.3 ATHEROSCLEROSIS

Atherosclerosis is a chronic, multifactorial and progressive disease that begins to develop already during adolescence [50]. It is typified by lesions, called plaques, in the arteries that are characterized by a fibrotic cap, inflammation, infiltration of immune cells, lipid accumulation initiating a lipid-rich core, cell death and fibrosis. However, the disease might not become clinically manifest until a plaque ruptures causing a thrombosis, which is the most common cause of coronary artery and cerebrovascular ischemic disease [51, 52].

1.3.1 The development of an atherosclerotic plaque

As described above, the arterial endothelium normally regulates the homeostasis; but, in response to certain circumstances and factors, *i.e.* turbulent blood flow, oxidized low-density lipoprotein (oxLDL), or pro-inflammatory mediators the endothelium becomes activated and more permeable, allowing entry and retention of cholesterol-rich LDL. Activated endothelial cells express leukocyte adhesion molecules that capture leukocytes (monocytes and T cells) in the circulation, which migrate into the artery wall. At the same time, endothelial cells secrete chemokines to attract monocytes, dendritic cells and T cells to the intima. Following this, monocytes in the intima are exposed to macrophage colony-stimulating factor produced by endothelial cells causing monocytes to differentiate into macrophages. Macrophages express scavenger receptors causing uptake of oxLDL; this cholesterol accumulation eventually turns the macrophages into foam cells [52].

As the lesion progresses, SMCs from the media migrate into the intima where they proliferate, produce extracellular matrix molecules and form a fibrous cap that covers the plaque. Underneath the cap reside numerous foam cells, of which some die and release their lipids. When the dead cells are not properly cleared away, accumulation of cellular debris and extracellular lipids is promoted, forming a lipid rich necrotic core. Clinical manifestations of atherosclerosis occur either through expansion of the lesion, which causes narrowing of the lumen, thus reducing the blood flow; or as a thrombus caused by plaque rupture, leading to rapid and complete obstruction of the vessel. Lesions that rupture are not necessarily the most obstructive ones; common characteristics of ruptured plaques are thin fibrous caps, and that they contain few SMCs but many macrophages. The infiltrating inflammatory cells within the plaque cause amplification of enzymes that degrade collagen and generate mediators triggering SMC death. Macrophages within the plaque also produce pro-coagulant tissue factor rendering the core thrombogenic [35] (figure 2).

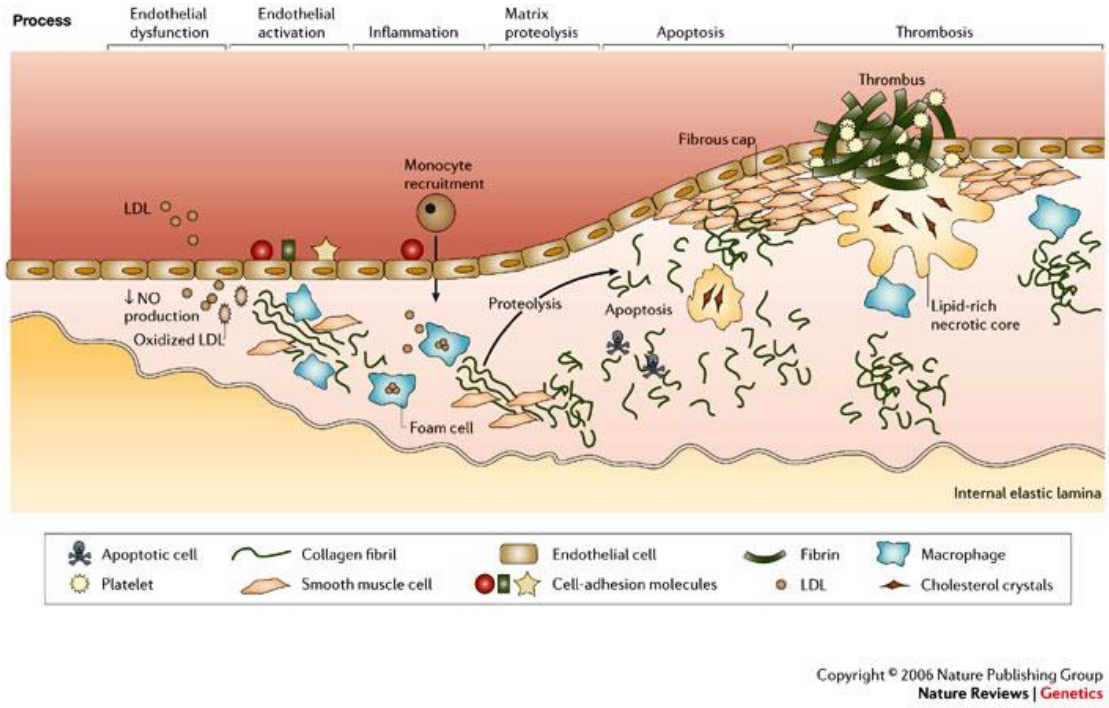

Figure 2. **The development of an atherosclerotic plaque and the cellular processes involved.** Reproduced with permission from Watkins, H. *et al*. Nat Rev Genet. 2006 Mar;7(3):163-73, Copyright ©Nature Publishing Group.

1.3.2 Endothelial dysfunction in diabetes

Endothelial dysfunction is characterized by an imbalance between vasodilating factors, vasoconstrictors, antithrombotic and prothrombotic factors, predisposing the endothelium to an atherogenic milieu [53]. When normal vascular endothelial function fails, it will change into a more activated phenotype which mediates adhesion of monocytes and platelets, increased synthesis of pro-inflammatory and pro-thrombotic factors, inflammation, trans-endothelial transport of LDL, increased oxidative stress, and inappropriate vasoconstriction or vasodilation [37, 42]. Endothelial dysfunction has been observed in T1DM, T2DM, in obesity, hypertension, smoking, and in patients with insulin resistance. The extent of endothelium-dependent vasodilation correlates in obese and insulin resistant subjects with their individual insulin sensitivity [42]. Endothelial dysfunction has thus emerged as an important early target for preventing atherosclerosis and cardiovascular disease [42, 54].

Pathological conditions such as hyperglycemia, hyperlipidemia, and inflammation, oftentimes seen in diabetic patients, might trigger superoxide ($O_2^{\cdot-}$) production, through activation of the NADPH oxidase [42, 55]. Superoxide quickly reacts with NO forming the very potent oxidizing species peroxynitrite ($ONOO^-$), which may oxidize the essential eNOS cofactor BH_4 or destabilize the eNOS dimer, causing uncoupling of eNOS. Uncoupled eNOS produces superoxide instead of NO, thus further enhancing the oxidative stress and decreasing NO bioavailability [55, 56]. Endothelial dysfunction is coupled to a decrease in NO, either by reduced synthesis or decreased bioavailability [57]. It has also been shown that bioavailability of NO is reduced in patients with T2DM [58, 59] affecting their endothelial-dependent vasodilation.

1.3.2.1 Glucotoxicity of endothelial cells

In diabetic patients, every cell in the body is exposed to abnormally high glucose concentrations; however, not all of them are adversely impacted by hyperglycemia. Endothelial cells are particularly susceptible to glucose-induced damage, probably because they express the glucose transporter (GLUT)-1 -- which is not sensitive to insulin -- which means that they cannot downregulate their glucose uptake when glycemia increases [60]. Hyperglycemia potentially promotes atherosclerosis by inducing many changes in the vascular tissue. The underlying mechanisms that have emerged map most of the pathological alterations seen in the vasculature of diabetic humans and animals. **1)** Advanced glycosylation end products (AGEs) are formed by the non-enzymatic glycosylation of proteins. These modified intracellular proteins and extra cellular matrix components have an altered function and interact abnormally with other matrix components and matrix receptors, respectively. Also, plasma proteins that are modified with AGEs interact with the AGE receptor (RAGE) on vascular cells, which induces the production of reactive oxygen species (ROS) and subsequent activation of the transcription factor nuclear factor (NF)-κB, causing pathological changes in gene expression [61, 62]. **2)** Hyperglycemia can also cause *de novo* synthesis of diacylglycerol (DAG), which activates protein kinase C (PKC). Activation of PKC might downstream lead to blood flow abnormalities, increased permeability of the endothelium, activation of the pro-inflammatory NF-κB, and oxidative stress by activation of NADPH oxidase. It may also cause thickening of the basement membrane in capillaries and over-expression of the fibrinolytic inhibitor, plasminogen activator inhibitor -1 (PAI-1), causing the vessel to be more susceptible to vascular occlusion [61, 62]. **3)** Hyperglycemia increases flux through the hexosamine pathway, which affects the activity of various enzymes, and of particular importance eNOS. Specific metabolites in this pathway have been demonstrated to bind to the Akt activation site of eNOS, thus inhibiting its activation [61, 63]. **4)** Hyperglycemia also augments oxidative stress by increasing the flux through the polyol pathway, causing a depletion of NADPH, an important co-factor for the antioxidant glutathione reductase [64]. The above mentioned theories have all emerged to explain the unfavorable effects of hyperglycemia on the endothelium; however, they were lacking a common element linking them together. A unifying hypothesis was therefore put forth by Brownlee in 2001 suggesting that oxidative stress, caused by overproduction of superoxide in the mitochondrial respiratory chain, is the common denominator upstream of these events [65].

1.3.2.2 Lipotoxicity of endothelial cells

Increased circulating free fatty acids (FFAs) are oftentimes seen in patients with T2DM [66]. FFAs, formed during lipolysis from triglycerides, are known to impair the endothelial-dependent vasodilatation in humans, and to decrease the bioavailability of endogenous NO resulting in impaired endothelial function [67]. One possible explanation for this is that FFAs have been shown to cause *de novo* synthesis of ceramides, which induce protein phosphatase 2A to co-localize with eNOS, leading to a reduced agonist-stimulated eNOS phosphorylation and NO production in endothelial cells [68]. FFAs have also been shown to activate IKK-β, a regulator of NF-κB. Upon activation impairs IKK-β insulin dependent insulin receptor substrate-1 (IRS-1), Akt and eNOS phosphorylation, and reduces NO production, probably by directly interfering with IRS-1 activity downstream of the insulin receptor [69]. As with hyperglycemia,

excess FFAs can also cause *de novo* synthesis of DAG that activates PKC, contributing to endothelial damage as described above. Elevated levels of FFAs might also cause lipid accumulation within endothelial cells; this can modulate membrane fluidity, proteoglycan metabolism and signal transduction [70]. In addition, increased levels of FFAs induce ROS, endoplasmic reticulum (ER) stress and apoptosis of endothelial cells, events that are supposed to be involved in, and contribute to, endothelial dysfunction, inflammation and atherosclerosis [71-73].

1.3.2.3 Oxidative stress

Diabetes is linked to oxidative stress. Oxidative stress occurs when there is an imbalance between oxidant production and the cellular antioxidant defense system. Oxidative stress, through ROS generation, causes oxidative damage to DNA, proteins and lipids [74]. One of the mechanism by which hyperglycemia and FFAs can induce oxidative stress appears to be through overproduction of the superoxide anion via PKC dependent activation of the NADPH oxidase [75]. Superoxide can in itself cause damage to cells, and as mentioned above, it might react with NO thus forming peroxynitrite [74]. Peroxynitrite might not only contribute to oxidative stress through oxidation of BH_4, but it can also oxidize other small-molecule antioxidants such as glutathione and cysteine. Apart from this, peroxynitrite can inactivate antioxidant enzymes, such as glutathione reductase, glutaredoxin and superoxide dismutase, as well as dimethylarginine dimethylaminohydrolase, which metabolizes the endogenous inhibitor of eNOS, asymmetric dimethylarginine (ADMA), thus further adding to the oxidative stress [76]. Another mechanism by which hyperglycemia and FFAs might induce oxidative stress is by overloading the mitochondrial respiratory chain due to excess electron donors (NADH, $FADH_2$) generated during the citric acid cycle. This may lead to leakage of electrons, which spontaneously form superoxide in the aerobic environment by reacting with oxygen, thereby increasing mitochondrial ROS production [56, 74].

An interesting hypothesis was recently put forth on the topic of oxidative stress and T2DM [77]. Contrary to the negative effects usually attributed to ROS formation, the author (the Nobel laureate James D Watson) suggests that an oxidative environment is needed for promoting the action of insulin. He speculates that ROS is necessary for proper folding of proteins, by providing a sufficient oxidative redox potential for formation of disulphide bonds in the ER. Without the correct folding, proteins will stay in the ER, initiating the unfolded protein response. He further alludes that antioxidants and metformin (due to its antioxidant effect) might lower the endogenous ROS levels below what is needed for proper disulphide bond formation, therefore abrogating the positive effect of exercise-induced ROS formation. This might then be the explanation of why antioxidant supplementation and metformin have been reported to prevent increased insulin sensitivity following physical activity [77-79].

1.3.3 Atherosclerosis and diabetes

It is generally agreed that people suffering from diabetes have approximately a two-fold increased risk of vascular disease, independent of other risk factors [80]. Patients with T2DM also have a greater plaque burden, a higher number of healed plaque ruptures and a larger necrotic core, compared to non-diabetic subjects [81]. Another study, systematically analyzing patients having undergone intravascular ultrasound,

showed that diabetic subjects had a greater burden of coronary atherosclerosis, an impaired compensatory remodeling of the vessel wall in order to accommodate the greater atherosclerotic burden and that the plaque progression was also more rapid, despite the use of established medical therapies, than their non-diabetic counterparts [82]. Why diabetic patients have a higher burden of atherosclerosis has not been fully elucidated. Oxidative stress is generally considered as a pathogenic mechanism for atherosclerosis. It is also considered to be one potential mechanism for the accelerated atherosclerosis observed in and associated with the metabolic syndrome, insulin resistance, prediabetes, and T2DM [62, 83]. Insulin *per se* and insulin resistance are also potential culprits in the atherosclerosis development seen in T2DM. Both *in vivo* and *in vitro* studies have shown that insulin, especially at high doses, promotes atherogenesis. Insulin resistance abrogates signaling through the insulin receptor, which impairs NO production, causing endothelial dysfunction and thus accelerating the atherosclerotic process [15]. Additionally, in insulin resistance there is a disproportionate increase in proinsulin, which has been reported to be associated with adverse cardiovascular effects [84, 85].

1.3.4 Interventions used to treat symptomatic atherosclerosis

Revascularization for patients with coronary artery disease is routinely performed throughout the world; percutaneous coronary intervention (PCI) being a commonly used procedure. With angioplasty, the narrowed or closed off vessel is reopened, using a thin catheter with a small inflatable balloon at the end. When in place, the balloon is inflated at the site of the lesion to compress the plaque against the artery wall, thus restoring blood flow. In order to increase the chances of keeping the vessel open a metal mesh tube, a stent, is often inserted into the lumen at the site of the lesion [86].

A successful revascularization in T2DM is in 25-30 % of the cases complicated by restenosis [87], which is a re-narrowing of the previously dilated vessel segment, and if the patient experiences symptoms a repeat revascularization is needed (figure 3). Restenosis usually reaches is maximum within the first 6-12 months after the procedure [88]. Restenosis is caused by vascular remodeling and formation of intimal hyperplasia, a vessel wall healing response involving migration and proliferation of SMCs in the intima, which leads to a re-narrowing of the lumen at the initial intervention site [89]. The process is also accelerated due to the loss of the endothelium, which normally cover the SMCs and inhibits their proliferation and migration [48]. The use of drug-eluting stents (DES) has reduced restenosis rates significantly. However, the re-intervention rate in diabetics with DES is around twice as frequent as that in patients without diabetes [90, 91]. Also, the risk of late in-stent thrombosis associated with DES is increased in diabetic patients [92-95]. The mechanism behind this phenomenon is not completely known, but a defective re-endothelialization, due to the non-selective anti-proliferative effect of the agent that is released from the stent, is suggested to be a common denominator for these cases [96].

Coronary artery bypass grafting (CABG) is another revascularization procedure in which a healthy vessel, harvested from another part of the body, is connected to the blocked coronary artery, the grafted vessel bypasses the lesion thus restoring blood

flow. It has been discussed whether or not diabetic patients benefit better from CABG than PCI with DES, a randomized controlled trial (the FREEDOM trial) specifically designed to compare these two methods has recently been published. Diabetics and patients with multivessel disease displayed a significantly reduced risk of all-cause mortality and myocardial infarction but a higher risk of stroke after undergoing CABG compared to PCI with DES [97].

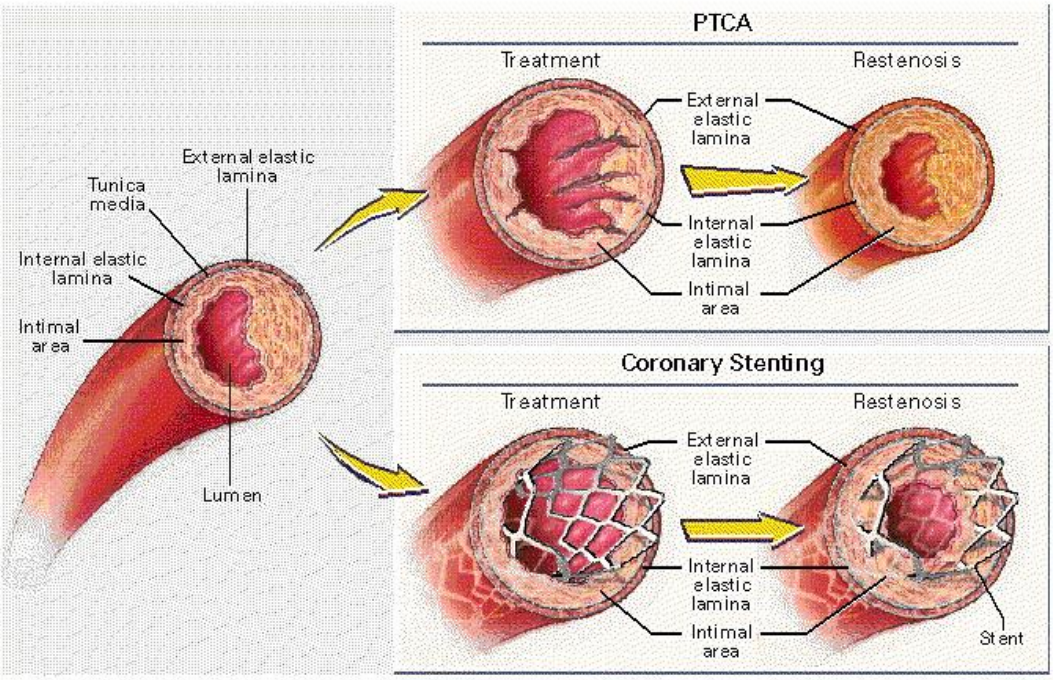

Figure 3. **Restenosis after percutaneous transluminal coronary angioplasty (PTCA) a.k.a PCI and coronary stenting.** Reproduced with permission from Bittl JA. N Engl J Med 1996;335:1290-1302, Copyright ©Massachusetts Medical Society.

1.4 GLUCAGON LIKE PEPTIDE-1

The concept that certain factors in the gut could influence glycemia was first postulated in the early 1900's [98, 99], and in 1932 Jean La Barre introduced the term "incretin", in order to describe these glucose-lowering, intestinal-derived factors [100]. In the mid 1960's, the methodology to measure insulin had improved and it could then be shown that an oral ingestion of glucose generates a higher increase in plasma insulin, when compared to intravenous infusion of an equal amount of glucose, again suggesting that gut-derived factors must be involved [101, 102]. This phenomenon was later termed "the incretin effect", and is estimated to be responsible for 20 to 60 % of the postprandial insulin secretion in healthy individuals [100, 103, 104]. Glucose-dependent insulinotropic polypeptide (GIP) was the first incretin to be identified in the 1970's; however, removal of GIP attenuated, but did not eliminate, the incretin effect, providing circumstantial evidence for the existence of another incretin. In the 1980's a second peptide was cloned and sequenced from the glucagon gene, *viz.* glucagon-like peptide-1 (GLP-1) [105], which was later shown to potentiate insulin secretion in both preclinical [106] and clinical studies [107, 108]. GLP-1 is produced and secreted from the enteroendocrine L-cells in the small and large intestine and is formed after posttranslational processing of proglucagon by

prohormone convertase 1/3 [109]. GLP-1 is released into the circulation in two equipotent forms, GLP-1(7-37) and GLP-1(7-36)amide, with GLP-1(7-36)amide being the most abundant in the circulation after a meal. Circulating plasma levels of GLP-1 range between 5-10 pmol/L in the fasted state but increases rapidly after a meal, reaching 15-50 pmol/L [110]. After its release, circulating GLP-1 is within minutes truncated by the serine protease dipeptidyl-peptidase-4 (DPP-4 a.k.a. CD26) at the alanine in the second amino acid position at the *N*-terminal to the metabolites GLP-1(9-37) and GLP-1(9-36)amide, which have 1,000 fold lower affinity for the GLP-1 receptor (GLP-1R) and lack insulinotropic activity [111, 112].

GLP-1 exerts its actions through the GLP-1R, a G protein-coupled receptor, first cloned from rat pancreatic islets [113]. GLP-1R expression has since then been reported in the pancreas, kidney, lung, central and peripheral nervous system, gastrointestinal tract, and the cardiovascular system. Then again, recently critique has arisen against the commercially available antibodies used to detect GLP-1R [114, 115]. Panjwani *et al* [114] detected immunoreactivity against proteins with a size consistent with the GLP-1R in lung tissue from GLP-1R$^{-/-}$ mice and when using PCR to detect expression of full length *glp-1r* mRNA transcript, no detection was seen in murine macrophages or hepatocytes, contrary to previous reports [116, 117]. These findings have raised concerns for studies reporting GLP-1R expression in cells and tissues without careful controls to thoroughly evaluate the presence or absence of full length *glp-1r* mRNA transcript and protein. This has engendered an interest in developing both new antibodies, that are more rigorously verified to be detecting the known GLP-1R [118], and other antibody-independent approaches to detect GLP-1R positive cells [115].

GLP-1 exerts pleiotropic effects in the body, both direct and indirect. It increases insulin and decreases glucagon secretion from the pancreas in a glucose dependent manner, thus controlling prandial glycemia. Beside these insulinotropic and glucagonostatic effects, GLP-1 has also been shown to increase, β-cell proliferation [119], insulin synthesis [106, 120] and to protect β-cells from apoptosis [121] in animal models and in human islets *ex vivo*. GLP-1 has also been reported to decrease appetite, gastric emptying and motility in the intestine. It also indirectly increases peripheral insulin sensitivity and decreases the glucose production from the liver. In addition and more related to the scope of this thesis, GLP-1 has been demonstrated to exert direct effects on the cardiovascular system [109, 122], which will be more thoroughly discussed below.

1.4.1 GLP-1 receptor agonists and DPP-4 inhibitors

GLP-1(7-36)amide has a half-life of approximately 2 minutes before it is degraded by DPP-4, which is ubiquitously expressed by many cell types, and also found as a soluble form in the circulation, thus complicating the application of GLP-1 for clinical treatment of diabetes [103]. Therefore have strategies to increase the presence of GLP-1 in the blood, either by using degradation resistant GLP-1R agonists (figure 4) or by inhibiting DPP-4 activity, been developed for the treatment of T2DM.

Exendin-4 is a naturally occurring peptide in the salivary gland venom of the Gila monster (*Heloderma Suspectum*). It was given its name since it was the fourth peptide to be found in an exocrine organ but shown to exert endocrine actions. Exendin-4

consists of 39 amino acids, which share 53 % homology with human GLP-1 [123, 124]. However unlike GLP-1, exendin-4 has a much longer half-life (~60-90 minutes). This since the alanine in the second amino acid position in the *N*-terminal region of GLP-1, which is site for DPP-4 cleavage, is replaced by glycine making exendin-4 resistant to degradation by DPP-4 [111]. Exendin-4 activates the GLP-1R and actually binds to it with greater affinity than native GLP-1 [125]. There is also a truncated form of exendin, exendin (9-39), which also binds to the receptor, but is unable to activate it, thus in effect serving as a GLP-1R antagonist [125]. Exenatide (Byetta®) is a synthetic form of exendin-4, which is approved for treatment of T2DM both in the U.S. and in Europe [126]. Exenatide is given twice daily as a subcutaneous injection and lowers both fasting and postprandial blood glucose; it has also been associated with a weight loss of 3-6 kg over 52 weeks. Since 2011, there is also an extended release formulation of exenatide available, exenatide LAR (Bydureon®), with a half-life of 4 days in the circulation. It represents the first once-weekly injectable anti-diabetic agent [126].

Liraglutide (Victoza®) is another GLP-1R agonist approved for treatment of T2DM. It shares 97% homology to the native peptide with an arginine 34 lysine substitution and a glutamic acid bound to a FFA addition to lysine 26. However, and unlike exendin-4, liraglutide is only partly resistant to DPP-4 degradation; but due to the FFA addition it can bind to albumin, shielding the DPP-4 cleavage site, thus extending its half-life to 10-14 hours [111]. Liraglutide, taken as a subcutaneous injection once daily, reduces both fasting and postprandial glucose levels and is associated with a weight loss of up to 2.5 kg after 30 weeks of treatment [111, 126].

Lixisenatide (Lyxumia®), a derivative of exenatide and administered subcutaneously once daily, was approved for treatment of T2DM in Europe in 2013. As expected, lixisenatide has effects on postprandial and fasting blood glucose levels in patients both as monotherapy and in combination with other anti-diabetic agents [127]. The pronounced effects of lixisenatide on postprandial blood glucose levels [128] have provided a clear rationale for combining it with basal insulin. This combination has been evaluated in three studies and been shown to be well tolerated. It significantly improved glycemic control, with a limited risk of hypoglycemia and a beneficial effect on body weight, either weight loss or no weight gain, which is important since insulin treatment otherwise is coupled to a body weight gain [127].

There are also other GLP-1R agonists under late-stage development, such as semaglutide, albiglutide and dulaglutide; however, none of them has as of yet been approved for treatment of T2DM [126].

Another way to address the issue with the rapid degradation of GLP-1 is to directly target DPP-4. Sitagliptin (Januvia®) was in 2006 the first DPP-4 inhibitor to be approved in the U.S. for treatment of T2DM [129]; since then several others, *i.e.* alogliptin (Nesina®), linagliptin (Trajenta®), saxagliptin (Onglyza®) and vildagliptin (Galvus®) have become commercially available. An advantage that DPP-4 inhibitors have compared to GLP-1R agonists is that they can be taken orally instead of as a subcutaneous injection. In contrast to GLP-1R agonists, DPP-4 inhibitors are almost weight neutral, which might be due to the modest increase in GLP-1 levels (15-25 pmol/L), compared to the pharmacological levels in plasma of the GLP-1R agonists [111]. Since numerous peptides have an alanine or a proline at position two, making

them susceptible to DPP-4 cleavage, it is hard to precisely pin point the peptide that mediate the glucose lowering effect of DPP-4 inhibitors. Notwithstanding this, in mice lacking both the GLP-1R and the GIP receptor, DPP-4 inhibitors lose their ability to lower blood glucose [130].

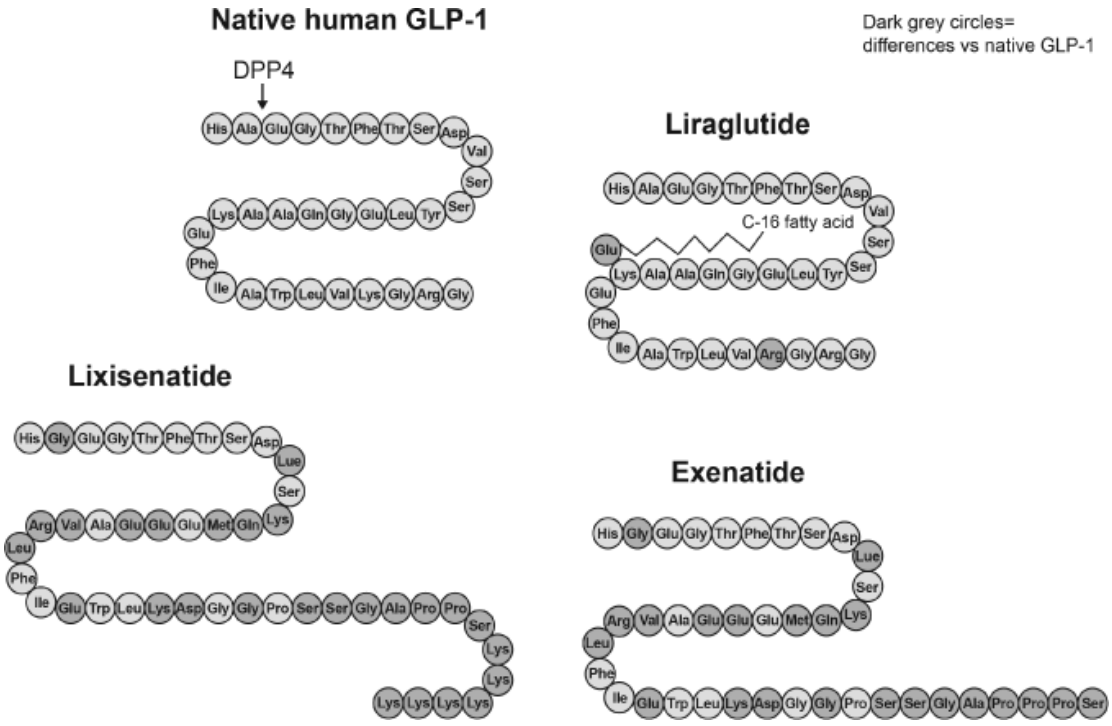

Figure 4. **Structure of native GLP-1 and GLP-1 analogues**. Reproduced with permission from Bolli GB., et al., Diabetes Obes Metab. 2013 Dec 24. Copyright ©2013 John Wiley & Sons Ltd.

1.4.2 Effects of treatment on vascular function

Over the years it has become evident that GLP-1, besides its insulinotropic actions, also exerts extrapancreatic effects, so also in the cardiovascular system [131-133]. The GLP-1R has been shown to be expressed both in the vasculature and the heart [118, 134, 135]. Since there is an uncertainty regarding the specificity of the GLP-1R antibodies, discussed above, the GLP-1R expression in the vasculature might need some additional verification. However, numerous studies have repeatedly shown effects of GLP-1 on blood pressure, heart rate, ischemia/reperfusion injury, coagulability, inflammation and on vascular endothelial function [136]. Adding to this, the GLP-1 metabolite GLP-1(9-36)amide, which has very low affinity for the GLP-1R, has been shown to induce vasodilation in rat and mouse arteries *ex vivo* [135, 137] and to increase the functional recovery from ischemia/reperfusion injury in the heart of both wild-type and GLP-1R$^{-/-}$ mice [135]. Remarkably, in the same study, exendin-4 was shown to have a partial cardioprotective effect in a GLP-1R-independent manner [135].

Richter *et al* were the first to demonstrate the GLP-1 exerted vasodilatory properties on rat artery rings. Upon removal of the endothelium, the relaxing effect of GLP-1 was

abolished, leading the authors to conclude that the effect was endothelium-dependent [138]. The vasodilatory response of GLP-1 was blocked in mesenteric arteries using L-NNA, a NOS inhibitor, further supporting the involvement of NO and the endothelium [135]. Since then this has also been observed in humans, in as much as a continuous infusion of GLP-1 improved endothelial dysfunction in T2DM patients with coronary artery disease compared to saline, measured by flow mediated dilation (FMD) [134]. Further supporting this, several other studies have shown similar effects of exendin-4 and DPP-4 inhibitors [139-144]. Even a single subcutaneous injection of exendin-4 improved postprandial endothelial dysfunction in newly diagnosed T2DM after a high fat meal [145]. However, there are also studies reporting no effect of GLP-1R agonists on endothelial function [146, 147] and even one study where the FMD was worsened after treatment with the DPP-4 inhibitors sitagliptin and alogliptin [148]. What distinguishes most of these studies from each other is the duration of the treatment. The majority of the studies showing positive effects on endothelial functions were either acute or no longer than four weeks. Studies showing no effect or even a worsened effect had a longer duration, making compliance to the medication a factor to consider.

Beneficial effects of GLP-1 and GLP-1R agonists on markers of endothelial cell dysfunction and cardiovascular risk have also been noted *in vitro*. GLP-1 and exendin-4 increase NO production and stimulate proliferation of human endothelial cells, through phosphatidylinositol-3 kinase (PI3K)/Akt-, protein kinase A (PKA)- and eNOS dependent pathways [149]. Liraglutide has been shown to reduce both the inflammatory cytokine tumor necrosis factor-α - (TNF-α) and hyperglycemia-induced expression of the fibrinolysis inhibitor PAI-1 and vascular adhesion molecules VCAM-1 and ICAM-1 [150]. Liraglutide also reduced TNF-α- and hyperglycemia-induced oxidative stress by inhibiting PKC translocation to the cellular membrane and thus reducing subsequent activation and superoxide production by NADPH oxidase [151, 152]. Liraglutide could also protect from TNF-α-induced apoptosis [151] ER stress and ER stress associated DNA damage [153]. Additionally, GLP-1 has been shown to protect from AGE-induced cell death [154].

GLP-1, GIP and exendin-4 have been shown to reduce monocyte adhesion, macrophage infiltration and atherosclerotic lesions in the vascular wall of $apoE^{-/-}$ mice, a commonly used animal model for studying the development of atherosclerosis [116, 155]. This effect was suggested by the authors to involve reduced foam cell formation, because oxLDL-induced cholesteryl ester accumulation was significantly decreased in macrophages harvested from GLP-1- and GIP-treated animals [155]. In contrast, others have reported that the GLP-1R agonist taspoglutide had no effect on the aortic plaque area in $apoE^{-/-}$ mice on a high fat diet, with a streptozotocin-induced hyperglycemia to simulate T2DM [114]. Thus the effect of GLP-1 on atherosclerosis development is still unclear, and more data on atherosclerosis associated outcomes, especially in diabetic humans, after long term treatment with incretin-based therapy is much needed.

GLP-1 has been suggested to exert direct effects on SMCs. In line with this our group noted that a vasodilatory effect of GLP-1 on femoral arteries *ex vivo* was evident even with inhibition of NOS and stripping of the endothelium [156]. Also, exendin-4 treatment has been shown to decrease intimal hyperplasia in a mouse model of

vascular injury, as well as in insulin-resistant rats *in vivo*, and to reduce SMC proliferation *in vitro* [157-159]. However the molecular mechanisms mediating this effect have not yet been elucidated.

Recently, two large clinical studies investigating the cardiovascular safety of the DPP-4 inhibitors alogliptin (EXAMINE) [160] and saxagliptin (SAVOR-TIMI 53) [161] were published. There was no reduced risk of fatal or non-fatal cardiac events, and in the SAVOR-TIMI 53 the rate of hospitalization for heart failure was even slightly increased for patients treated with saxagliptin. However, the patients were followed for a median of 18 months in the EXAMINE trial and a median of 25 months in the SAVOR-TIMI 53 study. As has been evident form other studies, such as the UKPDS, the effect on macrovascular complications might not be seen until after at least 10 years [31]. Other large studies, evaluating the cardiovascular outcome of GLP-1R agonists and DPP-4 inhibitors, are underway [162].

1.5 METFORMIN

Metformin (dimethylbiguanide) is an oral anti-diabetic agent of the biguanide family, and is currently used as first-line treatment of T2DM [163]. Metformin has a botanical origin in the *Galega officinalis*, which was used as an herbal remedy in medieval Europe to treat various conditions such as polyuria and plague. The first publication suggesting its anti-diabetic properties was published already in the 17th century [164-166]. *Galega officinalis* is rich in guanidine which has hypoglycemic properties in animals, but it was too toxic for clinical use. In 1922, the interest for guanidine derivatives led to the synthesis of several biguanides, including dimethylbiguanide [167], but it would take until the end of the 1950s before metformin (Glucophage®) was scientifically and clinically characterized, by Jean Sterne [165].

Treatment with metformin is associated with less hypoglycemic events and less or no weight gain when compared to insulin and sulfonylurea treatment. It is not completely known how metformin lowers blood glucose, but it decreases insulin resistance and reduces fasting insulin levels. Evidence from clinical and animal studies is however growing, suggesting that the primary function of metformin is to decrease hepatic glucose production, via decreased gluconeogenesis [163]. Metformin is believed to exert its pharmacological effects through AMP-activated protein kinase (AMPK) [168]. Interestingly, metformin induces secretion of GLP-1 from GLP-1-producing cells *in vitro* [169], increases total plasma GLP-1 levels *in vivo* [170] and 4 weeks of metformin treatment increased fasting plasma GLP-1 levels in diabetic subjects. Metformin treatment also augmented total plasma GLP-1 levels in both diabetic and non-diabetic subjects after an oral glucose load [171]. Moreover, metformin has been shown to protect human pancreatic islets *ex vivo* from functional abnormalities induced by glucotoxicity [172], lipotoxicity-induced impairment of glucose responsiveness and glucose metabolism [173], and apoptosis by reducing oxidative stress [174].

1.5.1 Effects of treatment on vascular function

In a substudy of the UKPDS, monotherapy with metformin decreased the incidence of macrovascular morbidity in overweight T2DM patients, compared to the control group, an effect that was not seen in the intensive treatment arm with insulin and

sulfonylurea. This outcome appeared to be independent of it glucose lowering property and it was suggested that it might be coupled to the decrease in PAI-1 levels seen with metformin treatment [175].

As mentioned above, metformin is believed to exert its pharmacological effects through AMPK activation [168]. AMPK has emerged as a new potential target in reversing endothelial dysfunction, both direct [176] and metformin-induced [177] activation of AMPK, has been demonstrated to lead to phosphorylation of eNOS, thus stimulating the release of NO, which is important for vascular function [168, 178]. In line with this, metformin improved endothelial function *in vivo*, by reducing ER stress, superoxide production and increasing NO bioavailability in mice on a high fat diet, an effect that involved the AMPK/peroxisome proliferator-activated receptor δ (PPAR δ) pathway [179]. Metformin was also shown to modulate oxidative stress caused by hyperglycemia. By decreasing DAG levels, PKC translocation to the cellular membrane was inhibited, and subsequent activation and superoxide production by the NADPH oxidase was thus reduced, an effect that was suggested to involve AMPK [152]. Activation of AMPK by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) has also been shown to protect from palmitate-induced apoptosis through suppression of ROS production in bovine aortic endothelial cells [72].

1.6 EFFECTS OF OTHER ANTI-DIABETIC AND CARDIOPROTECTIVE AGENTS ON ENDOTHELIAL CELL FUNCTION

1.6.1 Insulin

Insulin is the main regulator of glycemia in the body. It increases uptake of glucose into skeletal muscle and adipose tissue by regulation of GLUT-4 translocation to the cell surface. Besides glucose, insulin also promotes the uptake of FFAs and amino acids into cells. Insulin is an anabolic hormone, and it promotes synthesis and storage of carbohydrates, lipids, and proteins, while inhibiting their degradation and release into the circulation [180]. Insulin appears to have vasoactive effects in a NO-dependent manner [181, 182]. However, whether this occurs under physiological conditions is still a matter of debate [183, 184]. The insulin receptor is expressed on human coronary artery endothelial cells (HCAEC) [185] and activation of the receptor on endothelial cells leads downstream to PI3K/Akt dependent phosphorylation of eNOS at serine^{1177} and stimulation of NO production [186]. Insulin has also been reported to protect human umbilical vein endothelial cells (HUVECs) from palmitate-induced apoptosis through activation of the PI3K/Akt pathway [187].

Exposure of endothelial cells to FFAs impairs insulin-dependent phosphorylation of IRS-1, Akt and eNOS, through increased IRS-1 serine instead of tyrosine phosphorylation. Increased serine phosphorylation leads to a decreased PI3K activation, impairing the endothelial insulin sensitivity [69]. FFAs are not the only stimuli that may impair PI3K activation at the level of IRS 1 and 2; this phenomenon has been detected with TNF-α and angiotensin-II exposure and upon PKC activation. However, this resistance to insulin is somewhat “selective” because the other major pathway of insulin signaling, the mitogen-activated protein kinase (MAPK) pathway, is left unaffected. The MAPK, extracellular-signal-regulated kinase 1/2 (ERK 1/2) is responsible for production of the potent vasoconstrictor ET-1, causing an imbalance

between vasodilation and vasoconstriction, contributing to endothelial dysfunction [42, 188-190]. Insulin resistant individuals have elevated plasma ET-1 levels, and continuous infusion of triglycerides (Intralipid®) together with insulin increases ET-1 secretion in healthy subjects [191].

1.6.2 Sulfonylureas

Sulfonylureas render the β-cell more sensitive to glucose by binding to the sulfonylurea receptor (SUR)/Kir 6.2 subunit of the ATP-dependent potassium channel (K_{ATP}), which depolarizes the β-cell which causes insulin secretion. The K_{ATP} channels are not β-cell exclusive and can be found on other endocrine cells of the pancreas, in the heart, the brain, and in the vasculature on SMCs and the endothelium, thus spurring a concern whether binding of sulfonylurea to K_{ATP} channels in the heart and vasculature is harmful [192].

In the UKPDS, no increased morbidity and mortality was seen in patients treated with sulfonylureas compared to insulin treatment [23]. However, reports on vascular effects of sulfonylureas are contradictory. There are studies showing that the sulfonylurea glibenclamide exerts a pro-arrhythmic effect at reperfusion after a reversible ischemic event in conscious sheep [193] and has negative effects on vascular function in humans [194]. Sulfonylureas have also been coupled to an increased risk of congestive heart failure in a retrospective cohort study, when compared to metformin treatment [195]. Conversely, there are others that have shown an improved endothelial function in diabetic rabbits by gliclazide [196] and that gliclazide in comparison to glibenclamide attenuates the progression of atherosclerosis in humans, measured indirectly by the progression of intima-media thickness in the carotid artery [197]. Gliclazide has also been shown to protect from glucose-induced apoptosis of endothelial cells by reducing oxidative stress [198]. Also, glimepiride -- another sulfonylurea -- but not glibenclamide was shown *in vitro* to stimulate NO production in endothelial cells through PI3K/Akt dependent pathways [199, 200] and to reduce TNF-α-induced NF-κB activation [200].

1.6.3 Thiazolidinediones

Thiazolidinediones (TZDs) bind to and activate the PPAR subtype γ. PPARγ is ubiquitously expressed in the body and modulates the transcription of insulin-sensitive genes involved in the regulation of glucose and lipid metabolism in adipose tissue, skeletal muscle and liver. Treatment with TZDs improves insulin sensitivity and decreases circulating FFAs [201]. There are currently two TZDs on the market, rosiglitazone (Avandia®) and pioglitazone (Actos®).

PPARγ agonists modulate various inflammatory processes in the body, and TZDs have numerous anti-inflammatory effects [202]. Orasanu *et al* showed that pioglitazone decreased VCAM-1 expression on endothelial cells through modulation of NF-κB activity *in vitro* and *in vivo*, through a PPARα-dependent mechanism [203]. Exposure of endothelial cells to rosiglitazone has been shown to stimulate NO synthesis through an AMPK-dependent mechanism [204]. Concurrent with this, pioglitazone was shown to improve endothelial function in non-diabetic subjects with coronary artery disease, suggesting that pioglitazone exerts a direct effect on the endothelium [205].

Pioglitazone has been shown in a randomized controlled trial to not significantly decrease the large, combined primary cardiovascular end point, although -- and importantly -- the secondary clinical end point of stroke, myocardial infarction, and cardiovascular death was significantly improved, despite that these patients were on extensive cardioprotective background medication. However, an increased incidence of edema and heart failure was seen in the pioglitazone- treated group [206]. Edema is a well-known side effect of TZD treatment and is more often seen in patients with heart failure or with concomitant insulin treatment; however, the mechanism behind the development of TZD induced edema is not fully understood [207]. Further subgroup analyses, investigating the risk for recurrent MI and stroke, have shown protective effects of pioglitazone [208, 209].

In 2007, a meta-analysis including results from 42 widely different trials involving another TZD, rosiglitazone, Nissen *et al.* reported that treatment with rosiglitazone increased the risk for MI and death from cardiovascular causes [210]. As a consequence of this, rosiglitazone was revoked from the European market in 2010 and the U.S Food and Drug Administration (FDA) put it under selling restrictions; however, at the end of 2013, the FDA lifted these sanctions [211]. The meta-analysis by Nissen *et al.* has been criticized for overestimating the risk and being limited in its inclusion of data [212]. The Rosiglitazone Evaluated for Cardiovascular Outcomes and Regulation of Glycemia in Diabetes (RECORD) trial published 2009, which included 4,447 patients, could not confirm the results reported by Nissen *et al.* regarding the increased risk of cardiovascular morbidity [213].

1.6.4 Statins

Although cholesterol is important and necessary for human health, high levels of cholesterol in the blood, and especially LDL cholesterol, are associated with injury to arteries and cardiovascular disease. Statins inhibit the rate-limiting enzyme in the cholesterol biosynthesis, HMG-CoA reductase, leading to displacement of the natural substrate HMG-CoA, which effectively reduces serum cholesterol levels. Beside their primary function, statins might also exert direct (pleiotropic) effects on the endothelium [214]. Statins have been shown to be involved in the turnover of vascular cells; however, the data is somewhat discrepant. Some studies have shown that statins render endothelial and SMCs more susceptible to apoptosis, while others have shown the opposite or no effect [214]. Statins have also been implicated to attenuate plaque inflammation by reducing inflammation within the intima [215] and to influence atherosclerotic plaque stability by suppressing the expression of the pro-coagulant protein, tissue factor, and the extracellular matrix-degrading enzymes, matrix metalloproteinases (MMPs) *in vivo* and *in vitro* [216, 217] and to reduce macrophage content in experimental plaques *in vivo* [218, 219]. Treatment with statins in patients with preexisting cardiovascular disease and in diabetics without prior vascular disease has proven to be effective in decreasing mortality [220], but their value in primary prevention remains controversial [221, 222].

1.6.5 Angiotensin II receptor blockers

Hypertension is a well-established risk factor for cardiovascular disease. Angiotensin II receptor blockers (ARBs) are used to treat hypertension. ARBs bind to the angiotensin II receptor type 1 (AT_1), and by preventing the natural ligand, the vasoconstrictor angiotensin II, to bind they induce vasodilation. ARBs also exert effects on the vasculature, which are independent of its blood pressure-lowering properties or AT_1 receptor antagonism [223]. ARBs have been suggested to have anti-atherosclerotic effects, and have been shown to protect from development and progression of the lesions, and to promote plaque stability in experimental atherosclerosis *in vivo* [224-226]. *In vitro*, ARBs have been reported to protect endothelial cells against starvation and oxysterol (oxysterols constitute the major toxic component in oxLDL) induced apoptosis possibly by downregulating the gene expression of key components in the apoptotic machinery [223]. ARBs were also found to protect against hypoxia induced cell death, an effect that involved eNOS [227]. Treatment with ARBs increases NO release *in vitro* and ARBs have been shown to improve endothelial function in patients with the metabolic syndrome [228]. Candesartan, an ARB, was shown in the CHARM trial program to reduce cardiovascular morbidity and mortality in patients with chronic heart failure [229].

2 AIMS

The overall aim of this thesis was to investigate the role and mechanisms of drugs used for the management of type 2 diabetes on proliferation and apoptosis of cells in the vasculature under normal and simulated diabetic conditions both *in vitro* and *in vivo*.

Specific aims:

- To investigate the roles of anti-diabetic drugs on proliferation and lipotoxicity-induced apoptosis in HCAECs.
- To study the molecular mechanisms underlying the effects of a GLP-1R agonist and metformin on lipotoxicity-induced apoptosis of HCAECs.
- To investigate whether the proliferative and anti-apoptotic effects of a GLP-1R agonist noted *in vitro* on HCAECs are also operative *in vivo* to improve vascular repair and accelerate endothelialization in an animal model of vascular injury.
- To investigate the mechanisms by which a GLP-1R agonist influence proliferation and apoptosis of vascular smooth muscle cells *in vitro.*

3 MATERIALS AND METHODS

3.1 CELL CULTURE

Endothelial cell culture
Study I-III
Normal primary HCAECs, isolated from normal human coronary arteries (passage 5-13) and obtained from Clonetics (Lonza, Walkersville, MD), were grown in EGM-2 MV medium supplemented with 5 mM glucose, hydrocortisone, human epidermal growth factor , 5 % FBS, vascular endothelial growth factor, human fibroblast growth factor-B, R3-IGF-1, ascorbic acid and gentamicin/amphotericin-B at 37°C in a humidified atmosphere (5% CO_2, 95% air) as recommended by the supplier. Confluent cultures were detached by trypsin 0.025 %/EDTA 0.01 % and seeded onto tissue culture dishes and allowed to attach overnight before further investigations were performed.

Smooth muscle cell culture
Study IV
SMCs were isolated by enzymatic digestion from the aorta of adult Sprague–Dawley rats as previously described [230]. Cells were then cultured in F12/Ham medium supplemented with 10 % FBS (Gibco, Life Technologies, Carlsbad, CA), 50 µg/ml ascorbic acid, 100 units/ml penicillin and 100 µg/ml streptomycin. Confluent cell cultures were detached using 0.1 % trypsin and 0.8 g/l EDTA (SVA, Uppsala, Sweden) seeded onto tissue culture dishes and allowed to attach overnight before further investigations were performed. Cells between passages 4-7 were used for experiments.

3.2 VIABILITY

Study I
Cells were incubated in serum-deficient EGM medium containing 0.5 % FBS and
2 mM L-glutamine, in the presence or absence of the drugs for 48 h. Cell number was manually counted in a hemocytometer and cell viability assessed by Trypan blue exclusion.

3.3 [^{3}H]-THYMIDINE INCORPORATION

Study I and IV
For specific treatment conditions, please refer to each individual article.
Rates of [^{3}H]-thymidine incorporation into DNA were analyzed and used as a measure of DNA synthesis. Cells were pulsed with [^{3}H]-thymidine (1 µCi/ml) 8 h prior to the end of the incubation. Cells were then trypsinized, collected and homogenized through ultrasonication. Nucleic acids were precipitated in ice-cold 10 % trichloroacetic acid for 30 minutes on ice. The precipitate was washed with 10 % trichloroacetic acid and [^{3}H]-thymidine incorporation into DNA was measured using a microplate scintillation and luminescence counter (Wallac MicroBeta® Trilux, PerkinElmer) [231]. The protein concentration of the samples was measured using DC™ Protein Assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

3.4 DNA FRAGMENTATION

Study I-IV

For specific treatment conditions, please refer to each individual article.

Cell apoptosis was analyzed using the Cell Death Detection Kit plus (Roche Diagnostics) according the manufacturer's instructions. The Cell Death Detection Kit is an ELISA that measures cytoplasmic DNA-histone nucleosome complexes generated during apoptotic DNA fragmentation. Samples were measured at 405 nm and corrected for background signals caused by irregular microtiter plates or light scattering due to solid particles in the solution at the reference wavelength of 492 nm.

3.5 CASPASE-3 ACTIVITY

Study I and II

Cells were cultured to 90% confluence. After incubation in serum-deficient medium overnight, cells were pretreated for 1 h with the drugs or inhibitors, after which the incubation was continued for 24 h in the presence of 0.125 mM palmitate/0.25 % BSA or vehicle. Caspase-3 activity, a measure of apoptosis, was evaluated using the EnzChek® Caspase-3 Assay Kit (Molecular Probes®, Life Technologies) according the manufacturer's instructions. The assay is based on the 7-amino-4-methylcoumarin-derived substrate Z-DEVD-AMC, which yields a fluorescent product (excitation/emission ~342/441 nm) upon proteolytic cleavage by active caspase-3. All results were normalized to the protein concentration of the corresponding sample using DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA).

3.6 MEASUREMENT OF NITRIC OXIDE PRODUCTION

Study II

Direct measurements of NO release from HCAECs were performed using the cell-impermeable fluorescence indicator DAF-2 as described [232]. Cells were incubated in the presence or absence of palmitate with or without exendin-4 or vehicle in serum-deficient medium for 24 h. The cells were subsequently washed twice in Krebs-Ringer bicarbonate Hepes buffer containing 135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 10 mM HEPES, pH 7.4, followed by an incubation with 5 mM DAF-2 in 0.5 ml Krebs-Ringer bicarbonate Hepes buffer for 2 h, at 37 ^oC, using the eNOS substrate L-arginine (100 µM) as positive control. At the end of the incubation, supernatants were transferred into black microplates and the fluorescence was measured with the fluorescence microplate reader Infinite M200 (Tecan Group Ltd., Männedorf, Germany) at excitation wavelength of 488 nm and emission 515 nm. Results were normalized to the protein concentrations determined using BCA kits after the cells in each well were lysed in a lysis buffer containing 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 1% Triton X-100, pH 7.5.

3.7 MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES

Study II and III

For specific treatment conditions, please refer to each individual article.

Intracellular ROS levels were measured using Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, Life Technologies Europe BV) as previously described [233]. Briefly, the assay is based on 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), a fluorogenic marker that will be cleaved upon the presence of ROS. HCAECs were seeded into 6-well plates. Cells were then incubated for 24 h in the presence or absence of palmitate, vehicle, drugs or inhibitors. Cells were then washed with Hank's balanced salt solution before adding 25 µM carboxy-H2DCFDA to each well. After 30 minutes of incubation at 37 ^{o}C, excess probe was removed by washing the cells again with Hank's balanced salt solution. HCAECs were then lysed in PBS containing 1 % Triton X-100. Carboxy-DCF fluorescence in cell lysates was detected at an excitation/emission wavelength of 495/529 nm using a microplate reader (Tecan Group Ltd., Männerdorf, Switzerland). The fluorescence intensity was normalized against the protein concentration of each individual well.

3.8 GENE SILENCING

Study II

HCAECs were seeded into 100-mm dishes at a density of 2.5 × 10^5 cells per dish and incubated for 24 h at 37°C in complete medium. The cells were washed twice with culture medium without serum and supplement. Control siRNA/eNOS siRNA (10 nM) was mixed and incubated with SilenceMag according to the standard protocol (Oz Biosciences, Marseille, France). After incubation with a magnetic field for 15 min, the magnet was removed from the culture plate. 8-24 h post transfection, the media in the cell culture plate were replaced with complete medium containing 5 % FBS and then further incubated for 24 h. The cells were harvested and centrifuged at 200 *g* for 2 min to remove the supernatant.

3.9 ISOLATION OF TOTAL RNA AND REAL-TIME PCR

Study II

Total RNA was extracted from cells treated with eNOS siRNA and control siRNA, using Aurum Total RNA Mini kit, and reversely transcribed into complementary DNA with iScript^{TM} cDNA Synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). The PCR reaction mixture contained, in a final volume of 20 µl, 4 µl of cDNA, 10 µl of KAPA SYBR FAST qPCR master mix (Kapa Biosystems, Wilmington, MA) and corresponding primers [234]. The gene expression level was normalized to the housekeeping gene, β-actin.

Table 1. Primer sequences

Primer targets, human	***Forward primer***	***Reverse primer***
NOS3	5'-GTGATGGCGAAGCGAGTGAAF-3'	5'- CCGAGCCCGAACACACAGAAC-3'
ACTB	5'-AGCGGGAAATCGTGCGTG-3'	5'-CAGGGTACATGGTGGTGCC-3'

3.10 WESTERN BLOT

Study II-IV

Protein samples from cells were prepared for Western blot analysis using a modified RIPA buffer, containing 20 mM Trizma base, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.25 % Na-deoxycholate, 2 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF and 1% protease inhibitory cocktail (Sigma P-8340). After lysis the cell extracts were centrifuged at 4 ^oC and supernatants were transferred to another set of tubes. Protein concentration was determined by the DC™ protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA) and cell extracts were stored at -80 ^oC pending further analysis. Equal amounts of protein (15-30µg) were then mixed with reducing SDS-PAGE sample buffer, boiled for 5 min and proteins separated by electrophoreses against a pre-stained protein ladder on a 10-15 % polyacrylamide gel under denaturing conditions, followed by transfer to a polyvinylidene diflouride membrane. Immunoblot analyses were performed using antibodies that recognize phosphorylated ERK 1/2 (Millipore Corporatin, Billierica, MA), total ERK 1/2, phosphorylated Akt, total Akt (Santa Cruz Biotechnology, Heidelberg, Germany), phosphorylated AMPK, total AMPK, phosphorylated p38 MAPK, total p38 MAPK, phosphorylated c-Jun *N*-terminal kinase (JNK), total JNK, phosphorylated eNOS and total eNOS, cleaved caspase-3 (Cell Signaling Technology, Danvers, MA). Immunoreactive bands were detected using enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden), imaged with Molecular Imager ChemiDoc XRS and quantified with Quantity One software v. 4.6.5 (Bio-Rad Laboratories, Hercules, CA). Phosphorylation levels were determined after normalization with total forms of the protein. α-tubulin or staining of the membranes with Coomassie Brilliant Blue (Bio-Rad Laboratories) were then used to verify equal protein loading.

3.11 *IN VIVO* STUDY DESIGN

Study IV

The study was performed according to the guidelines of Karolinska Institutet and approved by the local animal ethics committee (N479/12). The number of animals in the experiment was chosen after power calculations based on a pilot experiment. 40 male Sprague-Dawley rats (~400 g) were used for the study. The animals were kept in groups through the whole length of the experiment in enriched cages. After arterial injury, the animals were randomized by a blinded operator for treatment either with exendin-4 (1 nmol/day) or saline. The treatments were given through a four week long continuous infusion by an osmotic ALZET® pump (2ML4) (DURECT Corporation, Cupertino, CA), placed subcutaneously on the back. Before surgery, after 2 weeks and at sacrifice, body weight and prandial blood glucose levels were monitored. At the same time blood samples were taken to analyze serum insulin and FFA levels.

3.12 ARTERIAL INJURY MODEL

Study IV

Carotid artery balloon injury and placement of osmotic ALZET® pump were performed under isoflurane inhalation anesthesia (4 % at induction and 1.5-2 % during the procedure). The rats were placed on a heating pad during surgery and ultrasound examinations and eye protection gel was used. After exposure of the left

carotid artery, an arteriotomy was performed in the external carotid artery and a 2F Fogarty embolectomy catheter was inserted into the left common carotid artery. The catheter was then advanced into the aortic arch, inflated and withdrawn with simultaneous rotation through the whole length of the artery while inflated. The passage was repeated three times in order to remove the endothelial layer. The external carotid artery was then ligated and the skin wound closed [235]. The animal was then turned onto its stomach, the osmotic ALZET® pump was placed subcutaneously on the back and the skin wound was closed. The animals were given Buprenorfin (Temgesic®) at 0.01 mg/kg body weight subcutaneously at the time of surgery, 12 and 24 h post procedure. The animals were controlled daily for pain signs and given additional analgesia when necessary.

3.13 ULTRASOUND VISUALIZATION AND MEASUREMENTS

Study IV

Ultrasound biomicroscopy has been shown to be a useful tool for the assessment of intimal hyperplasia and lumen diameter in the rat carotid injury model [235]. In study IV, a high-resolution ultrasound system (Vevo 2100, Visualsonics Inc., Toronto, Ontario, Canada), equipped with a MS700 transducer (up to 70 MHz), was used for *in vivo* evaluation of intimal hyperplasia and lumen diameter of the carotid arteries 2 and 4 weeks after the injury. The arteries were visualized longitudinally under isoflurane anesthesia (4 % at induction, 1.5-2 % during the examination) and the cine loops were saved for off-line measurements. Intima, media and whole vessel wall thickness were measured 10 mm proximal to the carotid bifurcation on the far wall of the artery in diastole using leading edge principle [235]. Arterial lumen was measured at systole and diastole just below and 10 mm proximal to the carotid bifurcation. The elasticity in terms of strain of the arterial wall was assessed just below the carotid artery bifurcation and calculated as percentage (systolic lumen diameter – diastolic lumen diameter) / diastolic lumen diameter * 100. The same observer made all the measurements in a blinded manner [236, 237].

3.14 SACRIFICE AND EVALUATION OF RE-ENDOTHELIALIZATION

Study IV

After 4 weeks of treatment, the re-endothelialization process was evaluated using Evans blue dye. Anesthetized rats were injected with 1 ml of 2 % Evans Blue, which was allowed to circulate for 15 minutes before animals were sacrificed by an overdose isoflurane and perfused with NaCl. The left common carotid artery was harvested and placed in saline, cut longitudinally and pinned open onto paraffin plates. Images of the vessels were taken using a dissection microscope (Leica EZ4D) for subsequent analysis of the re-endothelialization process. The total and the re-endothelialized areas (white areas, not stained with Evans Blue dye) of the artery were determined using the manual tracking tool in ImageJ software [238].

3.15 DETERMINATION OF SERUM PARAMETERS

Study IV

Prandial blood glucose levels were measured on saphenous vein blood at the same time in the morning, using a glucometer (OneTouch Ultra2; LifeScan, Milpitas, CA) at three time points, *viz.* study start (week 0), after 2 weeks and at sacrifice by week 4.

Serum levels of insulin were determined using a rat ultrasensitive insulin ELISA, (Mercodia, Uppsala, Sweden), according to the manufacturer's instructions. Serum concentrations of FFAs were measured using a "NEFA-HR(2) reagent", an enzymatic colorimetric method assay (Wako Chemicals GmbH, Neuss, Germany) according to the manufacturer's recommendation.

3.16 MASSON TRICHROME STAINING

Study IV
After the common carotid arteries were pinned and photographed for assessment of re-endothelialization, the samples were fixed with 4 % formalin for 24 h and then placed in 70 % ethanol. The samples were embedded in paraffin and cross-sectioned (5 µm). Sections at a distance of 10 mm from the bifurcation were stained with Masson Trichrome (Sigma Aldrich, St. Louis, MO) for subsequent morphometric analysis of the intima area. Image screening and photography of stained sections were performed using ScanScope CS slide scanner (Aperio, CA). Intima and media areas were blindly measured on five transverse sections from each animal using NDP-view software (Hamamatsu, Japan).

3.17 IMMUNOHISTOCHEMISTRY

Study IV
The common carotid arteries were fixed with 4 % formalin for 24 h and then placed in 70 % ethanol. The samples were embedded in paraffin and cross-sectioned (5 µm). In order to identify proliferating cells, the slides were stained using an antibody against proliferating cell nuclear antigen (PCNA) (DAKO, Glostrup, Denmark; 1:400) [239]. Three representative areas, evenly spaced, on each vessel were blindly counted to evaluate the percentage of PCNA-positive cells, using Image J software [238]. The total number of cells and the total number of PCNA-positive cells were then divided by the area to determine the total number of cells and the total number of PCNA-positive cells per area unit. Image screening and photography of stained sections were performed using ScanScope CS slide scanner (Aperio, CA) and NDP-view software (Hamamatsu, Japan).

3.18 STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. Student's t test was used to compare the difference between two groups. For multiple comparisons, one-way ANOVA or Kruskal-Wallis test (where appropriate), followed by the *post-hoc* tests Student–Newman–Keuls or Dunn's were used to determine statistical probabilities of differences between groups. Data was analyzed using the SigmaPlot v.11 software. A value of $p<0.05$ was considered statistically significant.

4 RESULTS AND DISCUSSION

Patients suffering from T2DM have a two-four times increased risk of a MI and stroke [20] and it is estimated that up to 80 % of the mortality in this group is due to a thrombotic event [240]. In people with T2DM and insulin resistance, endothelial dysfunction is a common finding and endothelial dysfunction is thought to be an early and important predictor for developing atherosclerosis and cardiovascular disease [42]. With the association between hyperglycemia and macrovascular complications not fully understood, together with the increasing global burden of T2DM [4], the importance of finding other strategies to improve cardiovascular outcome in T2DM patients has emerged as an urgent issue to be addressed. It has therefore been our aim to investigate how agents used for T2DM management directly, and not through their primary mechanism of action, affect the vasculature under both normal and simulated diabetic conditions.

4.1 PROLIFERATION OF HCAECs IS INDUCED UNDER NORMAL AND SIMULATED HYPERGLYCEMIC CONDITIONS

In **study I**, we set out to scan a number of agents that are currently on the market, and used within the treatment regime for T2DM, or in a developmental phase. We tested how these compounds affected viability and regeneration of HCAECs *in vitro*. We found that: insulin, metformin, rosuvastatin, pioglitazone, candesartan, glimepiride and the novel antidiabetic agent BLX-1002 could all significantly increase the viability of HCAECs when they were exposed to serum starvation. Insulin, metformin, rosuvastatin and BLX-1002 were also able to increase the proliferation of the cells under normal glucose conditions, as measured by [^{3}H]-thymidine incorporation rates. However, when the glucose concentration was raised to 11 mM, only insulin and BLX-1002 were still able to exert a positive effect on HCAEC regeneration. In the vasculature, damaged endothelial cells are usually rapidly replaced by proliferating healthy resident endothelial cells, thus maintaining endothelial integrity and protecting the vessel from development of atherosclerotic lesions [241]. However, hyperglycemia has been reported to induce proliferative dysfunction, oxidative stress and apoptosis of endothelial cells, thus making the already stressed diabetic vasculature even more susceptible to vascular complications [242-245]. Our findings that insulin and BLX-1002 stimulate proliferation of endothelial cells even under hyperglycemic conditions might therefore contribute to dampening or delaying the development of atherosclerosis. It may also contribute to preserving the endothelial integrity by inducing a more rapid healing response upon endothelial cell injury or apoptosis, hence protecting the vessel wall from thrombotic events [241]. We did not investigate the molecular mechanisms involved in the agents' proliferative effect on HCAECs, which is a limitation of **study I**. Notwithstanding this, insulin and rosuvastatin have previously been shown to activate the PI3K/Akt pathway, which is well known to be involved in survival and proliferation of cells [186, 246, 247]. Also BLX-1002, which is a novel TZD without known affinity for PPARγ, has been shown to signal through PI3K in β-cells [248]. Somewhat surprisingly we detected a slight but significant increase of [^{3}H]-thymidine incorporation in HCAEC in the presence of metformin [96].

Then again, this might be a reflection of metformin-induced NO formation [177], which is important for function, survival and growth of endothelial cells [48, 249].

4.2 HCAECs ARE PROTECTED FROM FFA INDUCED APOPTOSIS

In **study I, II** and **III** we investigated various agents' effect on apoptosis induced by the FFA palmitate. Diabetic patients, independent of their glucose control, have been shown to have more endothelial cells circulating in the blood than healthy controls, which might be an indication of vascular injury [250]. Elevated levels of FFAs are frequently found in patients with T2DM [251] and FFAs have been shown to impair endothelial function *in vivo* [67], in humans [252] and to induce apoptosis of endothelial cells *in vitro* [71], which might impair the integrity of the endothelium. Our data from **study I** showed that insulin, BLX-1002, metformin, pioglitazone and candesartan all attenuated the palmitate-induced cleaved caspase-3 activity and decreased the apoptotic DNA fragmentation. Again, in this scanning study, no molecular mechanisms were explored, but our findings are in line with others. Insulin, at supraphysiological concentrations, has been shown to protect from FFA-induced apoptosis through activation of the PI3K/Akt pathway [187]. BLX-1002 is a novel compound and not much is known about its effect on cellular signaling, but BLX- 1002 is able to activate PI3K and AMPK in β-cells [248], which have been demonstrated to convey anti-apoptotic signals in other cell types [72, 253, 254]. To the best of our knowledge, the effects of pioglitazone and candesartan on lipoapoptosis of endothelial cells have never been evaluated. However, both pioglitazone and candesartan have previously been shown to decrease caspase-3 activity and reduce apoptosis in endothelial cells exposed to TNF-α and hypoxia, respectively [227, 255].

In **study II** and **III** we continued to focus on apoptosis induced by the FFA palmitate but this time we tried to elucidate some of the molecular pathways involved. In **study II**, we investigated the effect of the GLP-1R agonist exendin-4. Previous findings from our group showed that exendin-4 activates the GLP-1R in HCAEC, leading to phosphorylation of eNOS and subsequent NO production [149]. Upon receptor activation, exendin-4 also increases cell proliferation through PKA-, PI3K/Akt- and eNOS-dependent pathways. Although GLP-1 has previously been shown to have anti-apoptotic effects in endothelial cells [151], cardiomyocytes [256] and neuronal cells [257], to this end and to the best of our knowledge, no one has studied the effect of GLP-1R activation on lipid-induced apoptosis of endothelial cells. Exposure of palmitate to HCAECs increased caspase-3 activity and apoptosis, which were dose-dependently reduced upon co-incubation with exendin-4. Even though exendin-4 is a known receptor agonist [125], we wanted to verify the involvement of the receptor, since there have been reports of differential effects of exendin-4 and GLP-1 on the vasculature, and that the GLP-1 metabolite GLP-1 (9-36) has vasoactive properties [135, 137]. These findings suggest that there might be a differential pathway involved or the existence of a second GLP-1R. We therefore introduced the receptor antagonist exendin (9-39) in our experiments. Co-incubation with exendin-4 and exendin (9-39) abolished the anti-apoptotic effect of exendin-4 and in addition GLP-1 (9-36) failed to protect the cells from lipoapoptosis, further supporting that the protective effect of exendin-4 is mediated via the canonical GLP-1R. Most effects of GLP-1R activation are downstream mediated through an increase in cAMP and subsequent PKA activation [258]; we therefore both blocked and activated PKA to confirm its involvement.

Blocking PKA inhibited the lipoprotective effect, whereas activation of PKA mimicked the effect of exendin-4, leading us to conclude that that PKA activity conveys the anti-apoptotic signals of exendin-4. Downstream of GLP-1R signaling, the PI3K/Akt pathway has been implicated in protection against apoptosis [259] and activation of PI3K/Akt has previously been shown to protect against lipoapoptosis of endothelial cells [187]. We therefore studied the effect of co-incubation with palmitate and exendin-4 on phosphorylation of Akt, and inhibiting PI3K and Akt with pharmacological inhibitors. Exendin-4 was able to increase Akt phosphorylation in the presence of palmitate and -- in further support of the importance of this pathway -- inhibition of PI3K and Akt abrogated the protective effect of exendin-4.

In **study III**, we wanted to elucidate the mechanism behind the lipoprotective effect of metformin found in **study I.** We hypothesized that the protective effect of metformin might be due to an AMPK-specific effect, since specific AMPK activation has previously been demonstrated to protect bovine aortic endothelial cells from lipoapoptosis [72] as well as HUVECs from hyperglycemia-induced apoptosis [260]. Our findings lend support to the notion that AMPK signaling can protect from apoptosis, since blocking AMPK led to a loss of metformin's protective effect, while pharmacological activation of AMPK mimicked the lipoprotection exerted by metformin. As expected, treatment with metformin led to an increased phosphorylation of AMPK, which was sustained even in the presence of palmitate. Surprisingly, palmitate, slightly but significantly, also increased phosphorylation of AMPK, as has previously been reported in myocytes [261]. This might be a reflection of an increased FFA oxidation caused by AMPK activation [178], and palmitate in combination with AMPK activity has been shown to enhance the oxidation even further [262]. Another plausible explanation for the increased phosphorylation of AMPK is that palmitate induce oxidative stress in endothelial cells [72], and oxidative stress *per se* has been shown to activate AMPK without strict AMP dependency [263, 264]. Interestingly, albeit not addressed in **study II**, recent reports on AMPK activation by GLP-1R activation have been published [152, 265, 266], raising the possibility that AMPK signaling was also involved in the findings of **study II**.

4.3 THE INVOLVEMENT OF eNOS AND ROS

Given that FFAs impair endothelial function [252, 267] and reduce NO bioavailability [268], and that NO has been shown to protect from apoptosis [269, 270], the importance of eNOS and NO formation was investigated in both **study II** and **III**, although more in detail in **study II**. Both exendin-4 and metformin are known to induce NO formation in endothelial cells [149, 177]. In **study II**, palmitate was unexpectedly found to induce NO formation and treatment with exendin-4 was coupled to an even further enhanced NO production. Adding further credence to eNOS as a protector against lipoapoptosis, both pharmacological inhibition and genetic silencing of eNOS abrogated the lipoprotective effect of exendin-4, suggesting that the protective effect of exendin-4 is eNOS-dependent. In **study II**, we unexpectedly detected an increased phosphorylation of eNOS by palmitate exposure, which was lowered upon concomitant exendin-4 treatment, whereas -- in **study III** -- palmitate decreased phosphorylated eNOS levels, an effect that was reverted back to control levels by metformin. At a first glance this may seem a bit contradictory, but it might be due to the differential experimental conditions used. In **study II**, we

investigated the effect of palmitate under starvation conditions, while in **study III** cells were kept in 5 % serum throughout the experiment. In **study II**, we believe that the increased eNOS activation is due to uncoupling of the enzyme, causing it to produce superoxide instead of NO and therefore contributing to the increased ROS levels seen with palmitate incubation. When cells are under stress from starvation, their access to its substrate L-arginine is limited, which can lead to uncoupling [271]. Also, serum-starvation *per se* induces ROS [272], which might cause an uncoupling of the enzyme due to oxidation of the cofactor BH_4 [55]. In line with this, supplementation of BH_4 in **study II** reduced both basal and palmitate-induced ROS production, to the same extent as exendin-4. On the other hand, the increased ROS production seen in **study III**, most likely does not originate from an uncoupled eNOS, but rather from a palmitate-induced NADPH oxidase activation [75], lipid oxidation or ceramide formation, all of which generate ROS [273]. Then again, this is purely speculative and has not been experimentally addressed, which is a limitation of **study III**. In **study II,** exendin-4 reduced FFA-induced ROS production and further increased NO production, by reducing the palmitate-induced eNOS phosphorylation, suggesting that exendin-4 can partially reverse eNOS uncoupling. This may seem paradoxical, but it has been shown that peroxynitrate, formed when NO reacts with superoxide, inhibits Akt-dependent Ser^{1177} phosphorylation of eNOS, but activates AMPK and increases AMPK dependent activation of the enzyme, which results in an increased superoxide production [264]. In **study II**, exendin-4 was able to increase Akt phosphorylation in the presence of palmitate, compared to palmitate alone. Adding to this, in **study III** we found that palmitate significantly increased AMPK phosphorylation. One can therefore speculate that exendin-4, in **study II**, partially restored eNOS uncoupling by increasing Akt-dependent eNOS phosphorylation [149], thus restoring the imbalance between NO and ROS generation. In **study III**, metformin was unable to reduce the increased ROS levels evoked by palmitate exposure, a somewhat surprising finding since others have shown that activation of AMPK reduces both FFA- and hyperglycemia-induced ROS formation in endothelial cells [152, 189]. However, as mentioned above, palmitate may increase lipid oxidation and ceramide formation, which both generate ROS [273]. Additionally, palmitate-induced lipid oxidation is even further increased upon simultaneous AMPK activation [262]. The unchanged ROS levels might therefore reflect an increased lipid oxidation, which contributes to metformin's lipoprotective effect. Also, the fluorescent probe used in both **study II and III** is not ROS species specific and have been used by others to detect NO [274]. Since we improved eNOS dysfunction in both **study II and III**, albeit in different ways, an increased NO formation might therefore interfere with our assay, leading to an overestimation of ROS generation in cells co-incubated with palmitate and exendin-4 or metformin. But then again, this is speculative since NO formation was not measured in **study III**.

4.4 THE MAPK PATHWAY IS INVOLVED IN MEDIATING FFA INDUCED APOPTOSIS

Both **study II** and **III** investigated the involvement of the MAPK signaling pathway, and especially the two pro-apoptotic MAPKs, p38 MAPK and JNK. Both these kinases are stress-inducible and are activated upon noxious external stimuli such as, however not exclusively, ROS, hypoxia, ischemia and cytokines [275, 276]. Incubation with palmitate caused p38 MAPK and JNK phosphorylation in both **study II and III**, but co-incubation with exendin-4 and metformin attenuated this activation; although metformin was not able to significantly reduce JNK phosphorylation, there was a clear

trend. p38 MAPK is suggested to be solely responsible for the increased apoptosis of HCAECs upon palmitate exposure [71]. This is contradictory to what we found in **study II** where we inhibited both p38 MAPK and JNK, and even then were we not able to completely prevent the lipid-induced apoptosis. In **study III**, upon inhibition of AMPK, metformin was no longer able to decrease the palmitate-induced p38 MAPK phosphorylation, suggesting that AMPK modulates mediators upstream of p38 MAPK. However, this unlikely occurs through reducing oxidative stress, which is a known inducer of p38 MAPK [275], as ROS levels were unchanged. Although blocking p38 and JNK led to a complete block of palmitate-induced ROS formation in **study II**, scavenging of ROS by the antioxidant Trolox® had no effect on apoptosis. This finding suggests, that in our experimental system ROS is not driving the lipoapoptosis, as been suggested by others [72]. We did not study the MAPK pathway upon inhibition of PKA, PI3K/Akt or eNOS, which of course is a limitation of **study II**, but in speculation, the PKA activator forskolin has been shown to inhibit p38 MAPK in HUVECs [277], and PKA activation has been shown to reduce JNK activation in cardiomyocytes [278], suggesting that exendin-4 may protect from lipoapoptosis by reducing p38 MAPK and JNK activation through PKA signaling pathways. As previously mentioned, GLP-1R activation can also induce AMPK phosphorylation [152, 266], suggesting that the AMPK pathway might be operative in modulating the activity of p38 MAPK and JNK also in **study II**.

4.5 GLP-1R ACTIVATION SELECTIVELY TARGETS SMCs IN A RAT MODEL OF VASCULAR INJURY

Restenosis after stent implantation has significantly decreased since the introduction of DES, but the use of DES is associated with an increased risk of late in-stent thrombosis due to a decreased re-endothelialization [279, 280]. This is caused by the non-selective anti-proliferative effect of the DES affecting all the cells in the vicinity, leading to impaired vessel healing and chronic inflammation, increasing the risk of thrombotic events [281]. Based on previous findings from our group, where we reported that exendin-4 increases endothelial cell proliferation *in vitro* [149] and the results from **study II**, where we discovered that exendin-4 could protect from lipoapoptosis *in vitro*, our aim in **study IV** was to test whether these findings also were operative *in vivo*. Increasing the re-endothelialization process would be desirable since it could speed up the healing process of the vessel wall, thus decreasing the risk of thrombosis and restenosis.

We used a non-diabetic rat model of vascular injury, where the endothelium is denuded causing a healing reaction in the vessel wall and the development of intimal hyperplasia [282]. We deliberately chose a non-diabetic rat model in order to evaluate if the effects exerted on the vessel were direct and not caused by glycemic and metabolic changes. Recently, there have been reports that exendin-4 can modulate intimal hyperplasia [157, 159] by reducing SMC proliferation [158]. Our findings are consistent with these reports; four weeks of treatment with exendin-4 in our injury model significantly decreased intimal hyperplasia and SMC proliferation formation *in vivo*, investigated both non-invasively with ultrasound and upon sacrifice with morphometric analysis and immunohistochemistry. We therefore set out to study the molecular pathways involved, mediating this effect. Our data show that the GLP-1R and subsequent cAMP/PKA signaling are both necessary for the reduced proliferation

of SMCs *in vitro* caused by exendin-4. This is in line with other research, showing that activation of cAMP/PKA can have differential effects in SMCs and endothelial cells [149, 283, 284]. PKA activation in SMCs led downstream to a decreased phosphorylation of ERK 1/2, whose activation is important for SMC proliferation [283], which is what we also detected in **study IV**. We also studied the effect of exendin-4 treatment on eNOS phosphorylation, since eNOS is expressed in SMCs, NO is well known to inhibit SMC proliferation and migration [48], and exendin-4 has previously been demonstrated to increase phosphorylation of eNOS and to induce NO formation in endothelial cells [149]. We found that treatment with exendin-4 increased phosphorylation of eNOS at Ser^{1177} in SMCs, which is coupled to an increased activity of the enzyme and subsequent, NO formation [285, 286]. Further supporting the involvement of eNOS, pharmacological inhibition of eNOS almost completely blocked the anti-proliferative effect exerted by exendin-4. Also in line with our findings, NO donors have been shown to reduce ERK 1/2 activation and migration of SMCs [287]. In their entirety our findings suggests that exendin-4 reduces SMC proliferation in a PKA-, eNOS- and NO-dependent manner.

On the other side of proliferation there is apoptosis, and cell death might function as a counterbalance [288] to the increased proliferation of SMCs seen after vascular injury. Remarkably, and contrary to what we found in **study II**, exendin-4 increased apoptosis of SMCs *in vitro* in **study IV**. This pro-apoptotic effect seems to involve an increased phosphorylation and activity of p38 MAPK, as phosphorylation of p38 MAPK was blocked upon GLP-1R antagonism. This is directly opposite of what we reported in **study II**, where exendin-4 treatment decreased p38 MAPK activity in endothelial cells. On the other hand, there have been several reports of cAMP/PKA-dependent p38 MAPK activation in many cell types, as thoroughly reviewed by Gertis *et al* [289], but also of PKA-dependent inhibition of p38 MAPK activation in HUVECs and a fibroblast cell line [277, 290], suggesting that cAMP/PKA signaling is cell type specific. The involvement of p38 MAPK in SMC apoptosis has previously been reported in both human SMCs [291] and airway SMCs [292]. The anti-proliferative and pro-apoptotic effect of exendin-4 might therefore work in concert to minimize the intimal hyperplasia, thus increasing lumen size and decrease the risk of restenosis.

Interestingly, data from our ultrasound visualization in **study IV** show that treatment with exendin-4 significantly improved arterial wall elasticity of the damaged vessel. This effect was observed at an injured but re-endothelialized part of the artery, indicating that the re-grown endothelial cells might be better functioning compared to vehicle-treated animals. The clinical significance of this finding is that arterial wall elasticity is a very important determinant of blood pressure, both systolic and diastolic, in the elderly population. Hypertension in this group contributes to various cardiovascular complications such as stroke, heart failure and aneurysm rupture [293, 294]. However, more research is needed to study the mechanisms behind this finding.

Although our data from **study II,** and our group's previously published study on proliferation of HCAECs [149] are promising, we were not able to detect any statistically significant stimulatory effect on vascular re-endothelialization by exendin-4 in **study IV**. The reason for this discrepancy is still unresolved but might relate to timing. Four weeks might either be too early or too late to discern re-growth of the endothelial cells, or we may be using a suboptimal method to analyze the re-

endothelialization process. Nevertheless and importantly, we demonstrate that exendin-4 treatment selectively targets the SMCs *in vivo*, since the endothelial cell regrowth after vascular injury was left intact. Such an effect is highly desirable, since, ideally, one wants to maintain a sustained inhibition of SMC proliferation, without suppression of endothelial cell coverage in healing a vascular wall injury.

Another important aspect of our findings is that, although local delivery of the antiproliferative agents eluted by the DES minimizes systemic toxicity, it does not protect from drug-drug interactions with other anti-diabetic agents that are present in the circulation. It was recently reported that in a rabbit model of iliac artery stenting, simultaneous systemic treatment with metformin decreased endothelial coverage of stent struts of zotarolimus-eluting stents and bare metal stents as compared to the groups treated with zotaroliums-eluting stents or bare metal stents alone [96]. Our results from both **study II** and **IV** suggest that treatment with exendin-4 should not further impair the healing process in the vessel. This is an important finding since diabetic patients have an increased risk of late stent thrombosis [92-95] and re-intervention after angioplasty [90, 91], and more T2DM patients are today being treated with GLP-1-based therapies, making drug-drug interactions something that needs to be taken into consideration. Germane to this, it may be wise to compare the effects of GLP-1R agonists with those of insulin, since insulin (and high glucose) reportedly increases proliferation of SMCs [295-297]. In the setting of vascular remodeling and intimal hyperplasia formation after a vascular wall lesion or stent apposition, a stimulatory effect of insulin on SMC proliferation would be detrimental as it may promote restenosis. Supportive of this, it has been described, in a study systematically analyzing patients having undergone intravascular ultrasound, that diabetic subjects receiving insulin had smaller lumen dimension than subjects not treated with insulin, despite similar plaque burden, suggesting an impaired compensatory remodeling due to insulin treatment. However, it is unknown if it is merely a reflection of a longer standing and more severe diabetes or a direct effect of insulin on the vessel wall [82].

5 CONCLUDING REMARKS

In conclusion, patients with T2DM have an increased risk of macrovascular complications, an increased atherosclerotic burden and an increased risk of complications after revascularization. Compared to microvascular complications, where the relationship to hyperglycemia is well supported, correcting solely hyperglycemia has so far not been successful in reducing cardiovascular events, highlighting the need for other strategies to improve the cardiovascular outcome for T2DM patients.

In this thesis, we provide evidence that drugs used for the management of diabetes exert beneficial effects on the cells in the vasculature under both normal and simulated diabetic conditions. We show in **study I** that agents commonly used in the management of T2DM can stimulate proliferation of endothelial cells under normal and hyperglycemic conditions *in vitro*, which might contribute to a delayed development of atherosclerosis, by replacing dysfunctional endothelial cells with healthy ones. It may also contribute to maintaining the endothelial integrity by inducing a more rapid healing response upon endothelial cell injury or apoptosis, thus protecting the vessel wall from atherothrombotic events. It may also be beneficial for diabetic patients undergoing revascularization and treated with DES, by improving the re-endothelialization process thus decreasing the risk of late stent thrombosis and restenosis.

Diabetic patients have been shown to have more circulating endothelial cells than healthy subjects, apparently independent of glycemia, indicating vascular injury. Since FFAs are elevated in T2DM patients, and FFAs impair endothelial function and lead to apoptosis of endothelial cells, we investigated whether commonly used anti-diabetic agents could protect HCAECs from lipoapoptosis. We also studied the molecular mechanisms underlying the protective effect of exendin-4 and metformin. We show in **study I** that a number of agents could lower FFA-induced caspase-3 activity and reduce apoptosis of HCAECs. In **study II**, we demonstrate that exendin-4 and GLP-1 protect HCAECs against lipoapoptosis, an effect that is mediated through the GLP-1R involving PKA-, PI3K/Akt, eNOS-, p38 MAPK-, and JNK-dependent pathways. We also report that palmitate activates eNOS, stimulate NO release, increases ROS production by uncoupling of the enzyme, and that p38 MAPK and JNK signaling pathways are involved in palmitate-induced ROS production and lipoapoptosis. In **study III**, we evaluated the protective effect of metformin on lipoapoptosis found in **study I**. We found that metformin can protect HCAECs from FFA-induced apoptosis through an AMPK-dependent mechanism, which recovered eNOS activity and decreased p38 MAPK signaling.

We continued to explore the protective effect of exendin-4 found *in vitro* in **study II** *in vivo* in **study IV**. We analyzed the effect of exendin-4 on endothelial cells and SMCs *in vivo*, in a rat model of arterial injury and tried to elucidate the mechanisms of the effects seen in *in vivo* on SMCs *in vitro*. Our findings show that exendin-4 selectively reduces proliferation and increases apoptosis of SMCs, effects that require the GLP-1R, PKA and eNOS. This selective effect of exendin-4, which is probably direct and not

mediated through glycemic effects, is therapeutically desirable as it minimizes neointima formation after vascular injury through inhibitory effects on SMCs, without impairing the re-endothelialization process. We also demonstrate that the arterial wall elasticity is improved, suggesting that even though we do not see an increased re-endothelialization, the re-grown endothelial cells might become better functioning with exendin-4 treatment.

To summarize, we show that drugs used in the clinical management of T2DM exert positive direct effects on the vasculature independently of their glycemic effects. This might be of clinical benefit for patients suffering from diabetes, and might serve to limit the adverse consequences of the macrovascular complications of T2DM, as dysfunction of endothelial cells is believed to contribute to premature development of atherosclerosis. Our findings might also be of therapeutic benefit for diabetic patients undergoing revascularization to treat atherosclerosis, since restenosis and late stent thrombosis are overrepresented and are potentially lethal complications among these patients.

6 FUTURE PERSPECTIVES

Since **study I** was only a scanning study it would be of interest evaluate the underlying mechanisms regarding the increased proliferation and protection from apoptosis seen with the agents investigated. It would also be of interest to expand **study III** and further explore the lipoprotective effect of metformin, to further evaluate the importance of eNOS, NO and the MAPK signaling pathway and also address why we see differential effects in ROS production compared to other studies. Is it due to an increased fatty acid oxidation? Moving on from **study IV**, it would be interesting to further study the functionality of the re-grown endothelial cells. For obvious reasons it would be highly relevant to study the effect of GLP-1 analogues and DPP-4 inhibitors in a diabetic animal model, but also in another vascular injury model, *i.e*. in a vein graft model to simulate a clinical setting where patients undergo bypass surgery, which can be hampered by restenosis. Lastly, it would be very interesting to take our findings into the clinical setting to evaluate whether there is a vascular benefit for patients undergoing revascularization to be treated with incretin mimetics.

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